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Role of membrane fusion protein Ykt6 in regulating epithelial cell-cell and cell-matrix adhesions.

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**ROLE OF MEMBRANE FUSION PROTEIN YKT-6 IN REGULATING EPITHELIAL
CELL-CELL AND CELL-MATRIX ADHESIONS**

This thesis is submitted in partial fulfillment of the requirements for the Master of Science
degree at Virginia Commonwealth University

By

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DEDICATION

To my beloved Aunt Mrs. Kamla Kattige
who is not here to see me successfully complete this endeavor

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List Of Abbreviations

%	-----	Percent
α	-----	Alpha
β	-----	Beta
γ	-----	Gamma
°C	-----	Degree Celsius
AJ	-----	Adherens Junctions
ATP	-----	Adenosine Triphosphate
ATCC	-----	American Type Culture Collection
BFA	-----	Brefeldin A
BrdU	-----	Bromodeoxyuridine
BSA	-----	Bovine Serum Albumen
Ca	-----	Calcium
CAS	-----	crk-associated substrate
CCAIM	-----	Carboxyl-terminal CAAX box sequence
DMEM	-----	Dulbecco's Modified Eagle Medium
DTT	-----	Dithiothreitol
ECM	-----	Extracellular Matrix

E-cadherin----- Epithelial Cadherin
 EDTA----- Ethylenediaminetetraacetic acid
 EGTA----- Ethylene Glycol Tetraacetic acid
 ER----- Endoplasmic Reticulum
 FA----- Focal Adhesions
 FAK----- Focal Adhesion Kinase
 GA----- Golgicide A
 GFP-----Green Fluorescent protein
 GTP----- Guanosine triphosphate
 HBSS----- Hank's Balanced salt solution
 HEPES----- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
 HGF----- Hepatocyte Growth Factor
 hr----- hour
 HRP----- Horseradish peroxidase
 JAM----- Junctional adhesion Molecule
 kDa----- Kilo Dalton
 MEM-NEAA----- Minimum Essential Medium-Non-essential amino acids
 Min----- minute
 ml----- Millilitre
 NaCl----- Sodium Chloride
 NSF----- N-ethylmaleimide-sensitive factor
 PAGE----- Polyacrylamide gel Electrophoresis
 PBS----- Phosphate Buffer Saline

p-pax----- phospho-paxillin
 PI----- Propidium Iodide
 PVDF----- Polyvinylidene fluoride
 RIPA----- Radioimmunoprecipitation assay buffer
 RISC----- RNA-induced silencing complex
 ROCK----- Rho-associated protein Kinase
 RPMI----- Roswell Park Memorial Institute Medium
 RNA----- Ribonucleic Acid
 SDS----- Sodium Dodecyl Sulfate
 SH----- Sufhydryl
 SNAP----- Soluble NSF-attachment proteins
 SNARE----- Soluble N-ethylmaleimide-sensitive factor attachment protein
 receptor
 TBS-T----- Tris-buffered saline-Tween
 TGN----- Trans Golgi Network
 TJ----- Tight Junctions
 ul----- Microliter
 wt----- Wild type
 ZO----- Zonula Occludin

Abstract

**ROLE OF MEMBRANE FUSION PROTEIN YKT-6 IN REGULATING EPITHELIAL
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By Supriya Krishnamurthy Joshi, Master of Science

This thesis is submitted in partial fulfillment of the requirements for the Master of Science degree at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

Thesis Director: Dr. Andrei Ivanov, Associate Professor, Department of Human and Molecular Genetics

Intercellular junctions and cell-matrix adhesions play important roles in the maintenance of epithelial integrity. Assembly and remodeling of the plasma membrane complexes are regulated by membrane trafficking and fusion. This thesis is aimed to elucidate the roles of an important membrane fusion protein, Ykt6, in the regulation of epithelial cell adhesion and migration. For the first time, we show that Ykt6 is essential for assembly of adherens junctions and tight junctions in human prostate epithelial cells. We also observed that Ykt6 negatively regulates

both collective epithelial cell migration and cell invasion into Matrigel. The effects of YKT6 on epithelial junctions involves expressional regulation of key junctional proteins, E-cadherin and claudin-4, whereas its effects on cell motility can be explained by antagonizing functions of junctional adhesion molecule-A. Overall, this study identifies YKT6 as a novel regulator of epithelial cell adhesions and motility.

INTRODUCTION

1.1. Structure of cell-cell adhesions and its role in the regulation of epithelial integrity.

Intercellular junctions, that are the most characteristic morphological features of mammalian epithelia, maintain the integrity of epithelial layers by mediating the interaction between adjacent epithelial cells. These plasma membrane structures create a barrier between adjacent cells so as to avoid the free paracellular passage of different molecules. Apart from maintaining epithelial integrity, the intercellular junctions are also essential for the establishment of apico-basal polarity within each cell that ultimately determines the absorptive or secretory phenotypes of the epithelial cells ^[1,2,3].

Mammalian epithelial cells can form several junctional complexes that include Tight junctions (TJs), Adherens Junctions (AJs), Desmosomes and Gap junctions ^[2,3,4]. The most apically located AJs and TJs are considered to be crucial regulators of paracellular barriers and epithelial cell polarity, and thus will be explained in greater detail. Fig: 1 shows the major integral and cytoplasmic proteins present at both of these junctions.

The adhesive properties of epithelial junctions are determined by specialized integral membrane proteins that include components of the adherens junctions such as E-Cadherin and Nectin as well as additional proteins like the Catenins (α and β). It also includes components of the tight junctions such as Occludins, Claudins and JAM-A as well as Zonula Occludins (ZO) ^[5,6,7,8,9,10,11].

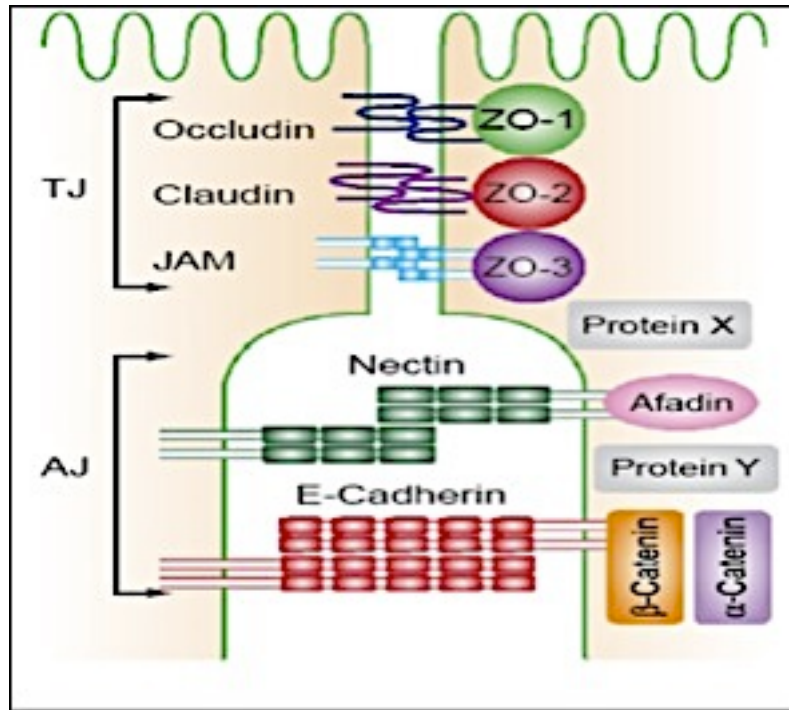


Fig 1: Major components of Tight Junctions and Adherens Junctions [Adapted from: Epithelial cell polarity and tumorigenesis: new perspectives for cancer detection and treatment. Coradini D, Casarsa C, Oriana S; Acta Pharmacol Sin, 2011, 32, 552-564.]

The Cadherins are an ancient superfamily of Ca^{+2} -dependent cell-cell adhesion proteins that have been very well characterized in recent times. Mammalian AJs contain a sub-set of these proteins known as the classical cadherins ^[13,14]. The classical cadherins are single spanned integral membrane proteins containing an extended amino terminal region, a transmembrane region and a cytoplasmic tail ^[14,15,17].

The cytoplasmic region of AJs is known as the 'Cytosolic plaque' and is composed of p120 Catenin, α -Catenin and β -Catenin. β -Catenin and p120-Catenin directly bind to the cytoplasmic

face of cadherins while α -Catenin binds to β -Catenin ^[16]. Catenins stabilize the cadherins and mediate their interactions with the actin cytoskeleton.

Occludin, a component of TJs, has four transmembrane domains, a long carboxyl-terminal cytoplasmic domain and a short amino-terminal cytoplasmic domain and has also been shown to be involved in the barrier function of tight junctions ^[18,21]. Claudins are the tetra spanning junctional proteins that give rise to the tight junction fibrils and thus play an important role in maintaining the integrity of tight junctions ^[18]. Next, the Junctional Adhesion Molecules (JAM's) are important regulators of the assembly and the barrier function of the TJ's ^[18,19,20,21]. These proteins have a unique PDZ-binding motif on their C-terminal ends that facilitate their interaction with the cytosolic plaque proteins.

The ZO proteins are the most important cytosolic plaque proteins of the TJs. These proteins contain multiple PDZ-domains as well as actin binding sites. Thus, they not only anchor the transmembrane tight junctional proteins but also mediate their interaction with the actin cytoskeleton ^[18].

Thus, the cytoplasmic plaque proteins of both the adherens junctions as well as the tight junctions enhance the adhesive properties of the epithelial junctions and regulate the biogenesis of AJ's and TJ's ^[6,7,8,9,12].

As per current literature, the AJ's and the TJ's are highly dynamic structures that undergo constant remodeling i.e. disassembly and reassembly ^[37-41]. The structure of junctional complexes depends on balanced turnover of AJ/TJ proteins that involves their removal from the plasma membrane via endocytosis and delivery to the plasma membrane via exocytosis ^[42,43,44].

Hence, the remodeling of junctions is highly important. While the endocytosis of the intercellular junctional proteins has been well documented by various groups, the role played by exocytosis in the remodeling of the junctional adhesion complexes is poorly understood [37,40,44,45, 46]. Mechanisms and the biological roles of trafficking of AJ and TJ proteins will be further explored in section 1.3 of this chapter.

1.2. Structure of cell-matrix adhesions and its role in the regulation of epithelial integrity.

Adhesion of epithelial cells to its Extracellular matrix (ECM) is an important determinant of tissue integrity that controls the formation of planar epithelial monolayers as well as complex three-dimensional tubules and glands [23,24]. Adhesion of cells to the matrix is also critical for the steady state cell migration in self-renewing epithelial layers in the gut and other organs. Furthermore, ECM adhesion regulates epithelial cell motility under pathophysiological conditions such as healing of mucosal wounds and metastatic dissemination of cancer cells [25,26,27,28]. ECM attachment of epithelial cells to its matrix is mediated by multiprotein complexes assembled at the cell base and known as Focal adhesions (FA) [29].

Fig. 2 below shows the important molecular components of focal adhesions.

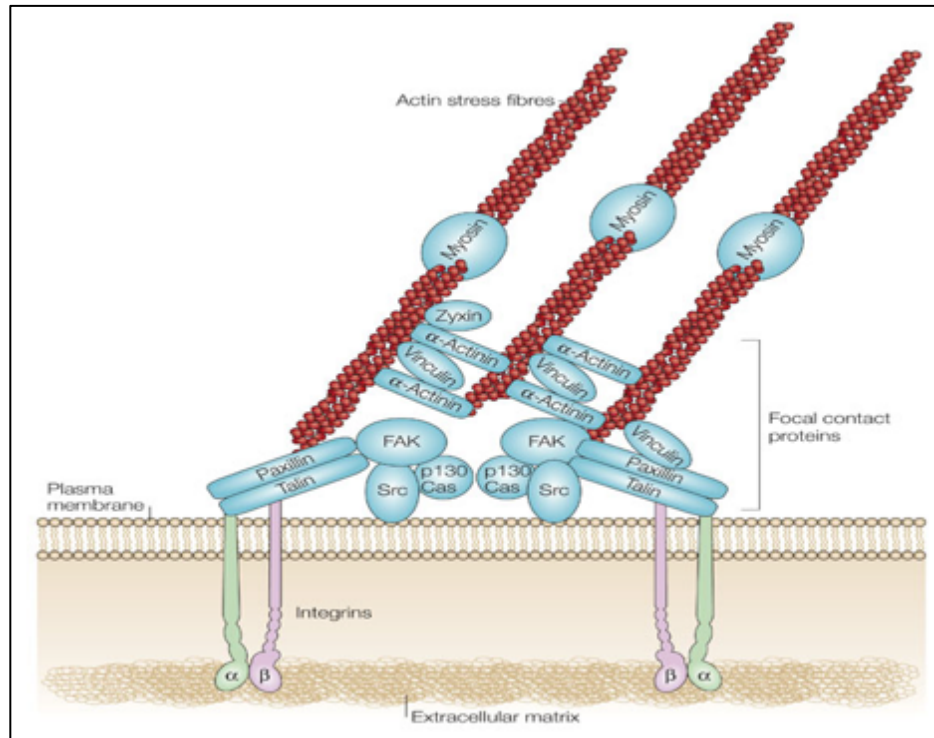


Fig 2: - Important components of Focal adhesions. [Adapted from: Focal adhesion kinase: in command and control of cell motility. Mitra, Satyajit K ; Hanson, Daniel A ; Schlaepfer, David D. Nature reviews. Molecular cell biology, 2005, Vol.6(1), pp.56-68]

FA are composed of multiple protein complexes that can be grouped into several different modules. Firstly, the transmembrane adhesive module that consist of the integrin family of proteins. Integrins family are heterodimeric transmembrane receptors that directly interact with extracellular matrix components ^[31,32]. Integrins function as non-covalently associated heterodimers of α and β subunits. Integrin subunits have large extracellular domains, a transmembrane region and a short cytoplasmic tail. The extracellular domains specifically link Integrins to the components of the extracellular matrix. β -1-integrin is one of the most important focal adhesion proteins that play a substantial role in maintaining cell-matrix contact as well as

regulating cell movement and migration ^[31]. The cytoplasmic scaffolding module involves a number of accessory proteins that regulates activation and clustering of the integrin proteins ^[33,34]. These include Paxillin, Vinculin, Talin, p130CAS etc. ^[30,33,34]. The signaling module involves several different kinases such as src kinase and Focal adhesion kinases (FAK) that phosphorylate different cytoplasmic scaffolds thereby triggering the assembly of FA cytosolic plaque ^[35,36]. Finally, the cytoskeletal module consists of a number of actin regulators that regulate actin polymerization and assembly of FA associated stress fibers.

Similar to the intercellular junctions, the FA are also dynamic in nature and undergo constant remodeling (i.e. assembly and disassembly). A simple functional model of cell migration implies that cells move by making protrusions at the migrating leading edge and retracting the trailing edge of the cell. FA are constantly formed at the leading edge of an actively migrating cell and are constantly disassembled at the opposite trailing edge thereby enabling cell movement ^[47,48]. This assembly and disassembly of the molecular components of the focal adhesion complex is essential for the continuity of focal adhesion biogenesis and their attachment to the extracellular matrix. Similar to its role in regulating remodeling of the epithelial junctions, vesicular trafficking is known to play a major role in FA assembly and disassembly. In section 1.3 below, we describe most important evidence that implicate vesicle trafficking in regulation of epithelial junctions and ECM adhesions.

1.3. Regulation of epithelial junction and ECM adhesions by vesicular trafficking.

Vesicular trafficking has two important components, endocytosis and exocytosis. The internalization (endocytosis) of cell-cell junctional proteins as well as cell-matrix adhesion

proteins has been well documented. For instance, AJ/TJ proteins were internalized when epithelial monolayers were challenged with various pathogenic stimuli ^[37,40,44,45,46]. Similarly, endocytosis is also known to be required for the recycling of the Focal adhesion proteins ^[61,62]. In contrast, the specific role of exocytosis in the remodeling and maintenance of cell-cell and cell-matrix adhesions is not very well documented. However, exocytosis has been implicated in the formation and maintenance of epithelial barriers as indicated by the steady state exocytosis of occludin and E-cadherin observed in various epithelial monolayers ^[49,50,51,52]. Moreover, disruption of exocytosis resulted in disruption of the reformation of the Tight and Adherens junctions ^[49,53,54,55,56].

Exocytosis can be defined as the directional transport of vesicles containing proteins or other molecules from the cell interior to the plasma membrane. It is a complex mechanism involving tethering, docking and finally fusion of transport vesicles with the target membranes ^[1,57,58,59,60]. This multistep process is regulated by a variety of accessory and signaling proteins that ensure the fidelity of the intermembrane interactions. Vesicular fusion, the final step of exocytosis, is also the rate-limiting step of exocytosis and results in the transfer of the transported molecule into its target membrane (e.g. plasma membrane) or its release from the cell.

Fusion of two phospholipid membranes cannot occur spontaneously and is mediated by the specialized SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein complex ^[58,60]. Specialized SNARE machinery is present on both the transporting vesicle (v-SNARE) as well as the target organelle (t-SNARE). The interaction of v-SNAREs and t-SNAREs bring the two membranes in close apposition ultimately leading to their fusion ^[63].

SNARE-mediated fusion events have been shown to be important for the assembly of epithelial tight and adherens junctions^[56,64]. Moreover various SNARE proteins have also been implicated in the regulation of integrin trafficking, Focal adhesion assembly and cell-matrix adhesion.

Hence, it is evident that continuous exocytosis is probably essential for the regulation of both cell-cell as well as cell-matrix adhesions, ultimately contributing to the efficient maintenance of epithelial integrity. Moreover, SNARE-mediated fusion events are an integral part of this complex mechanism. Therefore, the SNARE-mediated fusion mechanisms are described in greater detail in section 1.4 below.

1.4. The SNARE-cycle and its important functions.

Mature SNARE complexes are constantly disassembled and reused in order to maintain steady state exocytosis in various epithelial cells. This recycling of the SNARE-complex is mediated by N-ethylmaleimide-sensitive factor (NSF) and its adaptor proteins i.e. the soluble NSF-attachment proteins (SNAP)^[65,66,67,68,69]. Mammalian cells essentially contain α , β and γ -SNAP proteins out of which α and γ -SNAP are ubiquitously expressed in different cell types^[70]. The important roles played by these components in the SNARE cycle are depicted in Fig.3 below.

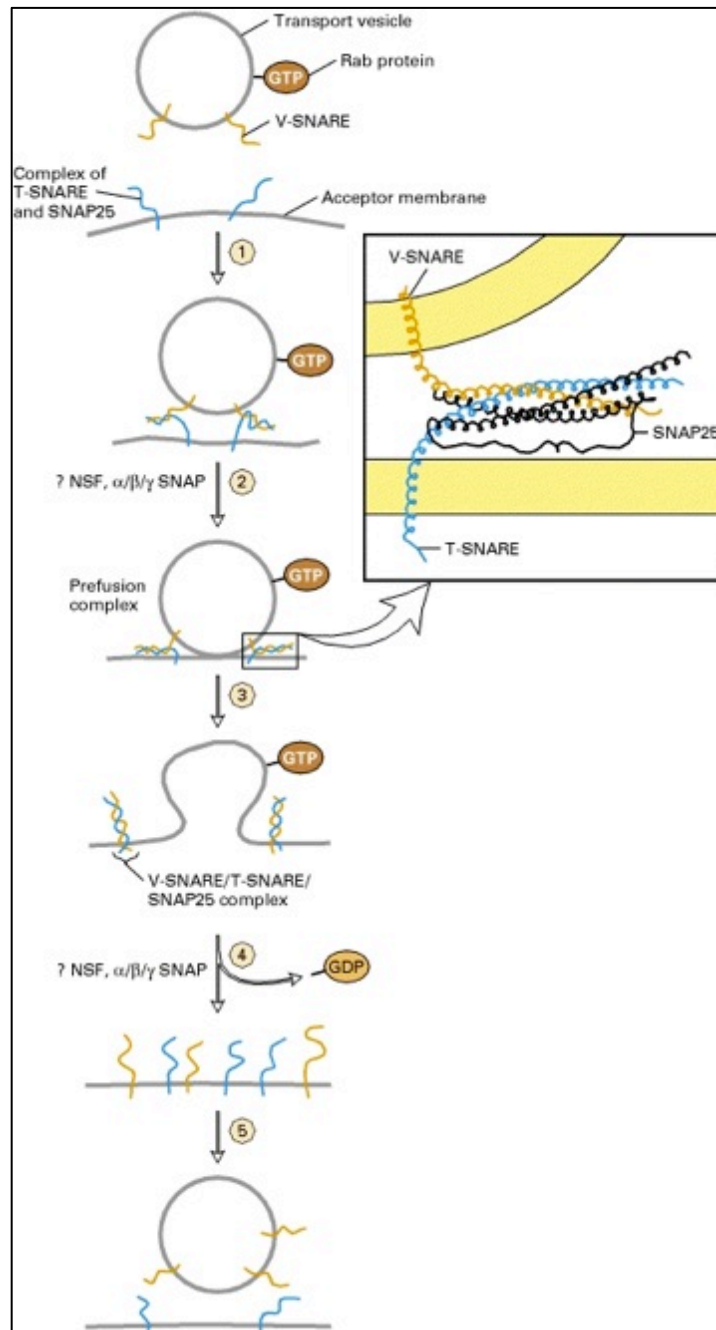


Fig 3: - Predicted mechanism of vesicular transport and vesicular fusion. [Adapted from- Molecular Biology of the Cell. 4th edition; Alberts B, Johnson A, Lewis J, et al. New York: Garland Science; 2002]

The figure above represents the predicted mechanism of the SNARE-mediated vesicular fusion. As per this mechanism, a transport vesicle binds to its target by the binding of the cytosolic domain of the v-SNARE protein to the cytosolic domain of the t-SNARE protein. This binding is mediated by accessory fusion proteins such as SNAP25 and leads to the formation of the pre-fusion complex. As shown in the figure above, four helices, two from SNAP25 and one each from the v-SNARE and t-SNARE proteins interact with one another to form a coiled coil that holds the two opposing membranes in close proximity. Energy required for the fusion of the two membranes is derived from the hydrolysis of an ATP or a GTP molecule. The formation of the pre-fusion complex can be selectively inhibited by N-ethylmaleimide, a molecule that reacts specifically with the sulfhydryl (-SH) group on the receptors and hence the complex is called as the SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex. Following fusion the v-SNARE/t-SNARE/SNAP25 complex is dissociated and recycled so that it can be reused for the next set of fusion events. This dissociation is mediated by the α , β and γ -SNAP proteins and is essential for the maintenance of steady state exocytosis in epithelial cells [72,73,74,75].

Recent studies have shown the SNARE protein complex and its accessory proteins to be important in the maintenance of cell-cell and cell-matrix adhesions. For instance, previous studies by various members of our lab and their collaborators have shown that the loss of the α -SNAP protein impaired golgi-dependent glycosylation and trafficking of β -1-integrin and also decreased the phosphorylation of FAK and Paxillin ultimately resulting in Focal adhesion disassembly [76]. Moreover, previous studies have also provided evidence that depletion of α -

SNAP can result in Golgi fragmentation and junctional disruption ^[1]. The functional consequences of α -SNAP depletion included a disruption in the golgi-dependent maturation of the β -1-integrin protein that requires Golgi-dependent glycosylation for its efficient processing and function. Moreover, reiterating this observation, various ‘Golgi toxins’ such as Brefeldin A (BFA) and Golgicide A (GA) were found to reproduce the effects caused by α -SNAP depletion, suggesting that Golgi-associated events may be responsible for the junctional barrier disruption observed in SNARE protein depleted cells ^[1].

The members of the SNARE protein family are made up of a number of small proteins with a very simple domain structure. Each SNARE protein consists of a characteristic SNARE motif, consisting of an evolutionarily conserved stretch of 60-70 amino acids arranged in heptad repeats. A C-terminal transmembrane domain and an N-terminal domain accompany this conserved stretch of amino acids. In specific SNAREs, the N-terminal domain has been shown to contain various profilin-like folds, also called the longin domains ^[66,85,86]. Events like Palmitoylation, Farnesylation, etc. can also post-translationally modify certain SNARE proteins ^[66]. These specific modifications may play a role in protecting the SNARE proteins from subsequent Ubiquitylation and degradation ^[66,80,87].

The structure described above stays true for majority of the existing SNAREs. However, there are important exceptions. Some SNARE groups lack the N-terminal domain while some lack the transmembrane domain. The Ykt6 protein is one such unique SNARE protein whose role in the regulation of junctional homeostasis has yet to be documented. Thus, this protein is the major protein of interest in our study and is described in greater detail in section 1.5 below.

1.5. SNARE protein Ykt6 and its functions:

The Ykt6 protein is an important SNARE protein that was initially described as an important regulator of the vesicular transport between the Endoplasmic Reticulum (ER) and the Golgi ^[80]. It is highly conserved from Yeast to man and was found to be associated with the Golgi, ER and Endosomal compartments ^[82,84]. However, the exact places of action and the biological roles of Ykt6 remain elusive since existing studies either support or dispute its role in the ER-Golgi trafficking pathway ^[83,84,92]. Ykt6 appears to be up regulated in certain types of cancer and its overexpression was shown to promote cell cycle progression ^[81,100]. Moreover, increased Ykt6 expression has been implicated in resistance to Docetaxel-an anti metastatic chemotherapeutic drug ^[81]. Overall, this data indicates that Ykt6 is likely to have multiple cellular functions that remain to be investigated.

The core structure of Ykt6 is similar to normal SNAREs, i.e. it contains the characteristic SNARE motif- an evolutionarily conserved stretch of 60-70 amino acids arranged in heptad repeats and an N-terminal domain. However, it is different from other SNARE proteins due to the replacement of the transmembrane domain by a carboxyl-terminal CAAX box sequence (CCAIM) ^[88]. Also, the N-terminal domain has been shown to contain various profilin-like domains called Longin domains with a hydrophobic motif which folds back on the SNARE motif giving rise to a closed ‘autoinhibitory’ conformation ^[84,89,90]. The Ykt6 protein like other SNARE proteins can also be post-translationally modified. For instance, the CCAIM terminal can be palmitoylated at the first cysteine and farnesylated at the second cysteine ^[84]. Another striking feature of Ykt6 is that it has been found to be localized to both the cytosol as well as membranes

i.e. it exists in both membrane bound as well as soluble cytosolic states ^[80,83,84,91]. The two lipid modifications mentioned have been found to be required for stable membrane association. Also, membrane bound Ykt6 has been thought to be the only form of Ykt6 that is functionally active. It is likely that the soluble form of Ykt6 is the closed ‘autoinhibitory’ form while the membrane attached Ykt6 is in the open conformation.

Figure 4 depicts the various different domains of the human Ykt6 protein- the characteristic SNARE motif, the N-terminal domain that consists of profiling like folds and the uncharacteristic C-terminal domain ^[84].

N-terminal Domain	SNARE Domain	CCAIM
-------------------	--------------	-------

KEY: - CCAIM- Carboxyl-terminal CAAX box sequence

Fig 4: - Domains of Human Ykt6 [Adapted from:- Localization and activity of the SNARE Ykt6 determined by its regulatory domain and Palmitoylation. Fukasawa, M., Varlamov, O., Eng, W.S., Sollner, T.H., and Rothman, J.E. Proc.Natl.Acad.Sci.USA,2004.

Similar to other SNARE proteins, Ykt6 has also been shown to act through the formation of a SNARE complex. The various known binding partners of Ykt6 include bet1, syntaxin 5 and GS28 and this SNARE complex has been shown to participate in late stage ER-Golgi transport ^[92].

Apart from being a highly debatable SNARE protein in terms of its localization and functions in different epithelial cell lines, the role of Ykt6 protein in the regulation of cell-cell and cell-matrix

adhesion still remains uninvestigated. Hence, in this study, we attempt to elucidate the role of the Ykt6 protein in the regulation of junctional homeostasis, cell adhesion and cell migration.

2. MATERIALS AND METHODS

2.1. Cell Culturing

Cultures of different epithelial cells were maintained in standard T-flasks. Du145 and other epithelial cells were plated onto 6-well plates and 24-well plates and allowed to grow overnight in nutrient media until a confluency of 50-60% is achieved. The cells were further transfected as per the methods described below. The various essential components of the nutrient media used for cell growth is given in the table below.

Cell Type	Basic Medium	Source	Serum Used	MEM-NEAA	Pen-strep	Additions
Du145	RPMI	Invitrogen	Fetal Bovine serum (10%)	NA	5 ml	HEPES-7.5ml Pyruvate-5ml
HeLa	DMEM	ATCC	Fetal Bovine serum (10%)	5 ml	5 ml	HEPES-7.5ml
HEK293T	DMEM	ATCC	Fetal Bovine serum (10%)	5 ml	5ml	HEPES-7.5ml

2.2. siRNA-mediated protein knockdown

Cells cultured on 6-well plates were transfected with gene specific siRNA in order to obtain transient protein knockdown. Ykt6 gene silencing was obtained using specific On-Target⁺ siRNA from ThermoScientificBio. The siRNA is specifically designed to reduce off target effects. As per the mechanism stated by the manufacturer, the sense strand is modified to prevent interaction with RISC-complex and thus favors anti-sense strand uptake while the anti-sense strand is modified to destabilize off-target effects and enhance target specificity (Manufacturer's manual, ThermoScientificBio). For Ykt6 gene silencing, a siRNA On-Target⁺ pool or four individual duplexes were initially used to minimize possibility of off target effects. Two most effective siRNA duplexes were selected for subsequent experiments. For depletion of other proteins of interest such as E-cadherin, Paxillin and JAM-A, specific siRNA from ThermoScientificBio were used. All knockdowns were performed as per manufacturers protocol. Control siRNA duplex#2 obtained from ThermoScientificBio was used as control in all experiments.

2.3. Cell lysates

Cell lysates were obtained on the fourth day post-transfection. Cells were first washed with HANK's+ balanced salt solution (HBSS) and then scraped from the 6-well plates using RIPA cell lysis buffer (Tris 20mM, NaCl 150mM, EDTA 2mM, EGTA 2mM, Na DOX 1%, SDS 0.1%, Triton 1%). The lysis buffer was supplemented with protease inhibitor cocktails 2 & 3 (1:200) and phosphatase inhibitor (1:100) [both obtained from Sigma] to protect protein integrity and protein phosphorylation. The samples were then homogenized using a Dounce homogenizer.

The homogenized samples were then centrifuged at high speed (20 minutes at 15000G, 4°C) to separate the protein containing solution from cell debris. The samples were then mixed with an equal volume of 2X SDS sample buffer containing 0.1M Dithiothreitol (DTT) and boiled for 5 mins. The samples are then stored as aliquots in -80°C refrigerator.

2.4. Western Blotting (Immunoblotting)

Total cell lysates were separated by Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) with 10-20ug of protein loaded into each well. The separated proteins were then transferred by standard electroblotting technique onto Nitrocellulose or Polyvinylidene difluoride (PVDF) membranes. PVDF membranes were used for proteins with a molecular weight of less than 20kDa while Nitrocellulose membranes were used for all the other proteins. The membranes were blocked and then treated with monoclonal or polyclonal antibodies directed to the protein of interest at dilution ranging from 1/500-1/1000. 5% Non-fat dry milk was used as a blocking and diluting reagent for all proteins except for phosphoproteins. 3% bovine serum albumen (BSA) was used as a blocking and diluting reagent for Phosphoproteins. The overnight incubation of the membrane with the primary antibody was followed by 3 washing steps with TBS-T (Tris-buffered saline-Tween-20) to remove excess antibodies (15 mins each wash). The membranes were then incubated with Horseradish peroxidase (HRP) conjugated goat anti-Mouse or anti-Rabbit secondary antibody at a concentration of 1:5000. The treatment of the membrane with the secondary antibody was subsequently followed by several 3 steps with TBS-T to remove excess antibodies (15 mins each wash). The membranes were finally developed using standard enhanced chemoluminescence reagents from GE Healthcare following the

manufacturers protocol. Images were processed on standard X-Ray films using the SRX-101A medical image processor from Konika Minolta.

2.5. Immunofluorescence Labeling and confocal microscopy.

Epithelial cells cultured on collagen-coated coverslips were transfected with either control or Ykt6 specific siRNA. On the fourth day post-transfection, the cells were fixed in 100% methanol for 20 minutes at -20°C . After fixation, the cells were blocked for 1 hr at room temperature (in the dark) using 1% Bovine Serum Albumen (BSA). The primary antibody dilutions (1:300) were first prepared using BSA as the diluent and the cells were then stained using appropriate primary antibody combinations for 1 hr. This was followed by three 5 min washes with BSA. The cells were then stained with the appropriate secondary antibodies (Diluted in BSA at 1:1000) for 1 hr. this is again followed by three 5 min washes with BSA to remove any excess protein. The coverslips were mounted on glass slides using ProLong Gold anti-fade reagent from Invitrogen (Life technologies). Stained cells were observed using Zeiss LSM 700 Laser Scanning Microscope (Zeiss Microimaging Inc.; Thornwood; NY). The Alexa Fluor 488 and 568 signals were imaged sequentially in frame-interlace mode to eliminate cross talk between channels. Image analysis was conducted using imaging software ZEN 2011 (Carl Zeiss Microscopy Inc.; Thornwood; NY) and Adobe Photoshop. Multiple images were captured from each slide.

2.6. Wound Scratch Assay

Cells were transfected on 6-well plates using the methods described above. On the third day post-transfection, a 20ul pipette tip was used to make a vertical thin scratch wound in the most confluent area of the cell monolayer. The plates were then washed using nutrient media and each

well was marked for study. Images of the scratch were then taken at 0 hours, 8 hours and 24 hours. After 24 hours the level of wound closure was quantified using the TScratch software.

2.7. Cell Spreading Assay

Cells were transfected on 6-well plates using the methods described above. On the 4th day post-transfection, the cells were trypsinized and counted. Next, 10^7 cells were seeded onto 24 well plates pre-coated with collagen I ECM. The plates were then incubated for 1 hr to allow the cells to attach and spread on the ECM. The cells were then examined under high power microscope to determine spreading area of the attached cells.

2.8. ECM Adhesion Assay

Cells were transfected on 6-well plates using the methods described above. On the 4th day post-transfection, the cells were trypsinized and counted. Next, 10^7 cells were seeded onto collagen coated 24-well plates. The plates were then incubated for 30 mins to allow cell attachment to the ECM. Non-attached or poorly attached cells were removed by one stroke of 1ml pipette up and down and extensive washing with cell culture medium. The cells are then fixed with Methanol for 10 minutes. Fixed cells are further stained using Eosin Y (10 min) followed by Azure A/Azure B (10 min). The stained cells were then counted under a high power bright field microscope.

2.9. Invasion Assay

Cells were transfected on 6-well plates using the methods described above. On the 3rd day post-transfection, the cells were trypsinized, resuspended into serum free media and counted.

Approximately, 3×10^7 cells were loaded into the upper chamber of the Bio-coat matrigel coated membrane-inserts. The upper chamber was filled with serum free media while the lower chamber of the matrigel plates was filled with normal cell culture medium containing 10% FBS as a chemoattractant. Cells were then allowed to migrate through matrigel overnight at 37°C. Thereafter, plates were washed with HANK's + Balanced salt solution (HBSS) and are then fixed with Methanol for 10 minutes. Fixed cells were stained using Eosin Y (10 minutes) followed by Azure A/Azure B (10 minutes). The upper surface of the matrigel was wiped clean with a cotton bud in order to remove the cells that did not invade the matrigel. The invaded labeled cells were counted using a bright field microscope.

2.10. Cell proliferation assay.

The FlowCelleTM Bivariate Cell Cycle Kit for DNA replication Analysis by Millipore is used for the cell proliferation assay. First, cells were transfected with control or Ykt6 specific SiRNA on 6-well plates using the methods described above. On the day 3 post-transfection, the cells were trypsinized and resuspended in a 5cm petri plate and allowed to grow overnight. The cells were then fixed and permeabilized using the fixation and permeabilization buffers respectively from the kit. The cell pellet obtained from the step above is treated with DNaseI (diluted to 300ug/ml in Assay buffer provided in kit) for 1 hr. The cells are then washed with assay buffer to remove the DNA denaturation buffer. Further centrifugation is carried out to remove the assay buffer so that only the cell pellet remains. Next, stain the cells with anti BrdU (Bromodeoxyuridine)-antibody direct conjugate provided in the kit (1 hr). This is again followed by a wash with the assay buffer to remove any excess BrdU antibodies (2 washes). The cells are then stained with

Propidium iodide (PI)/RNase solution for 30 minutes. Note that a sample stained only for BrdU and only stained for PI should be kept as a control. The stained cells are then counted and analyzed using the Gauva Flow cytometer.

2.11. Plasmid DNA Transfection using the TransIT[®]-293 reagent.

Plasmid DNA transfection was performed in a similar manner to the previously described SiRNA transfection method. The only difference lies in the transfection reagent being used. For this transfection, the TransIT[®]-293 transfection reagent (MirusBio), which is specialized for plasmid DNA transfection is used to overexpress Wild-type ykt6 and its mutants in HEK 293T cells.

2.12. Antibodies and Plasmids

Ykt6 wild-type and mutant plasmids were obtained from Jesse Hay at the University of Montana. The following primary polyclonal (pAb) and monoclonal (mAb) antibodies were used to detect matrix adhesion, trafficking, signaling and junctional proteins: anti β 1-integrin mAb (Novus Biologicals, Littleton, CO); anti α 5-integrin , pAb (EMD Millipore, Billerica, MA); anti-NSF, total paxillin, mAbs (BD Biosciences, San Jose, CA); and vinculin mAbs (Sigma-Aldrich, St. Louis, MO); anti-phospho-paxilin , total FAK, phospho-FAK and GAPDH pAbs, src pAb, phosphor-src pAb (Cell Signaling, Danvers, MA); Zo-1 pAb, JAM-A pAb, Claudin-4 mAb (Invitrogen); E-cadherin, β -catenin mAb, p120 Catenin (BD Biosciences); α -catenin mAb(ABCam). The Alexa Fluor secondary antibodies against rabbit and mouse were obtained from Life technologies.

3. RESULTS- PART I

3.1: Ykt6 localizes in different cellular compartments.

In order to examine possible functional roles of YKT6 in epithelial cells, we first investigated intracellular localization of this protein. HEK293 human embryonic kidney cells were transfected with myc-tagged Ykt6 and on day 2 post-transfection, the cells were fixed and dually- labeled for the myc-tag (red) and β -catenin (green). The Ykt6 protein was localized in different cellular compartments being enriched in close vicinity to the nucleus and at cell-cell junctions (Figure 5: Top panel). This localization was clearly different from localization of another ER-Golgi resident v-SNARE, Sec22b, which demonstrated a restricted perinuclear ER labeling (Figure 5: bottom panel). These results indicate existence of different intracellular pools of YKT6 and unexpected enrichment of this SNARE protein at the plasma membrane of epithelial cells.

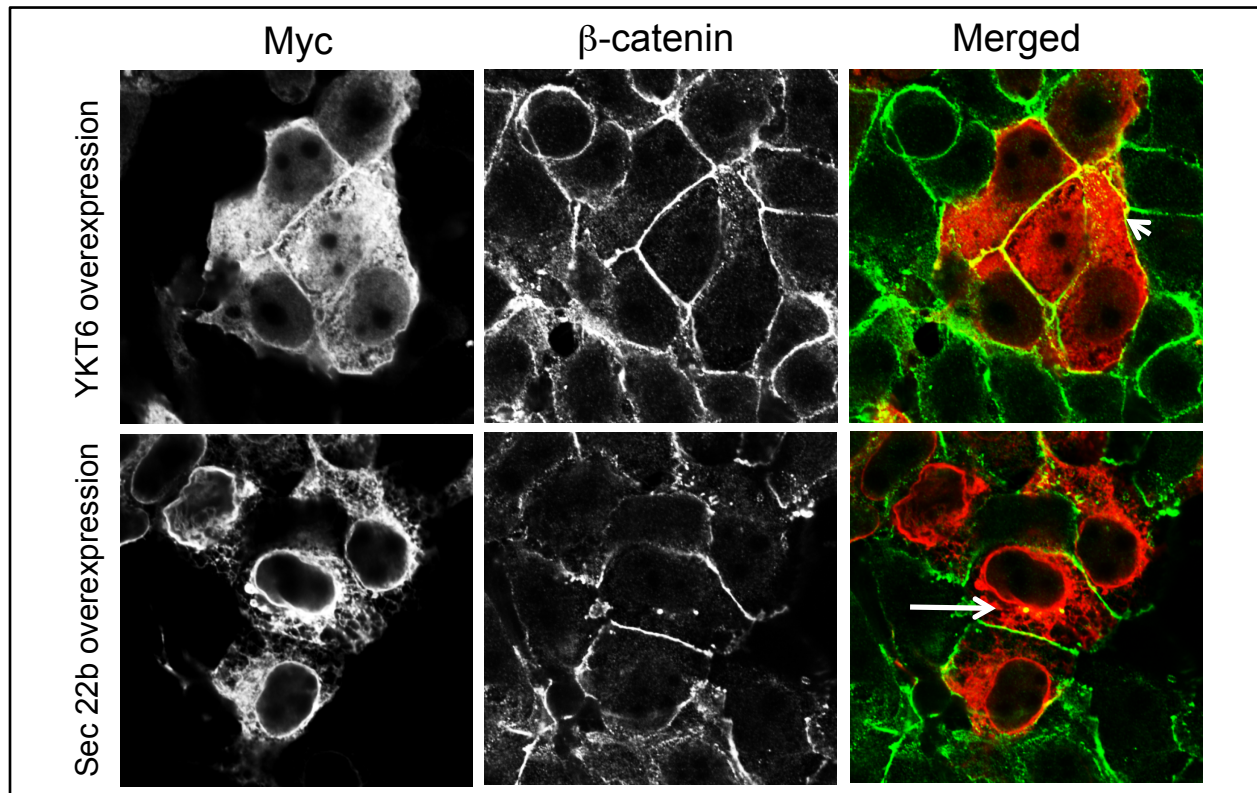


Figure 5: Different localization of Ykt6 and Sec 22b in epithelial cells. 293 T HEK cells were transiently transfected with either Myc-tagged YKT6 (Top panel) or Myc-tagged Sec 22b (Bottom Panel) constructs. Cells were fixed and dual labeled for Myc tag (red) and Beta –catenin (green) 48 h post-transfection. Note a significant colocalization of exogenous YKT6 with cell-cell junctions (arrowhead). By contrast, Sec22b is predominantly localized in the perinuclear compartment (arrow).

3.2. Ykt6 can be efficiently depleted in different epithelial cells using RNA interference.

In order to investigate possible functional roles of Ykt6 we sought to downregulate its expression in epithelial cells using RNA interference (RNAi). To do that, we used either YKT6 specific siRNA SmartPool or individual siRNA duplexes (D9-D12). Figure 6 shows that transfection of A549 and Du145 epithelial cells with SmartPool or individual duplexes dramatically decreased YKT6 expression in both epithelial cell lines.

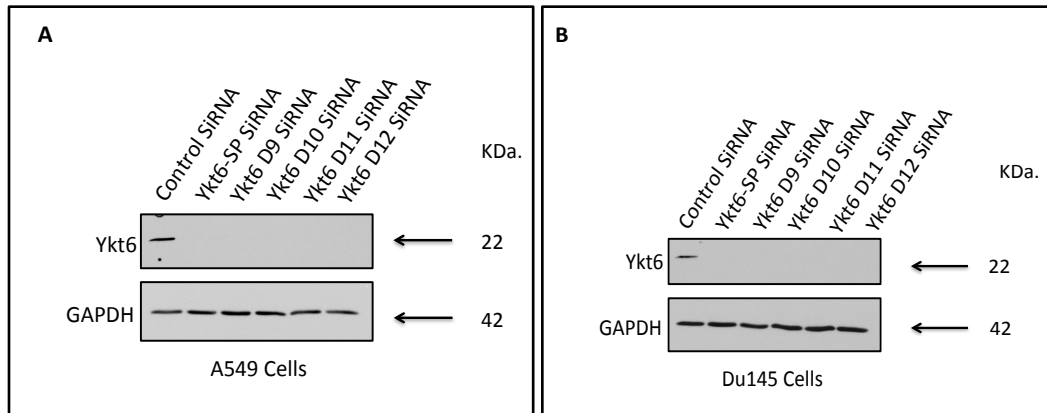


Figure 6: Ykt6 is efficiently depleted by corresponding siRNAs in different epithelial cell lines. 6A. Immunoblotting performed to check for successful knockdown of Ykt6 in A549 Cells. Cell lysates were obtained on the fourth day post-transfection. 6B. Immunoblotting performed to check for successful knockdown of Ykt6 in Du145 Cells. Cell lysates were obtained on the fourth day post-transfection. [Note: - ‘D’ indicated Duplexes]

3.3. Loss of Ykt6 disrupted cadherin based junctions and intercellular adhesions in Du145 cells.

We next investigated the effect of YKT6 depletion on the integrity of epithelial junctions. Since our preliminary study observed formation of both AJs and TJs in DU145 cells, we used this cell line to study effects of YKT6 knockdown on epithelial junctions. Integrity of AJ was evaluated by immunolabeling Du145 cells with E-cadherin, Cadherin-6, and β -catenin, whereas TJ integrity was examined by immunolabeling the cells with JAM-A. Confocal microscopy demonstrated characteristic ‘chicken wire’ labeling of AJ and TJ proteins in control siRNA-transfected DU145 cells (Figure 7, arrows). This labeling was markedly disrupted in DU145 cells transfected with two different YKT6-specific siRNAs. Indeed, these cells showed significant decrease in the labeling intensity of E-cadherin at the areas of cell-cell contacts. Furthermore, Cadherin-6, β -catenin and JAM-A translocate from cell-cell contact zones into the

cytosol (Fig. 7 arrowheads). Together, this data strongly suggests that depletion of YKT6 triggers disassembly of epithelial AJs and TJs.

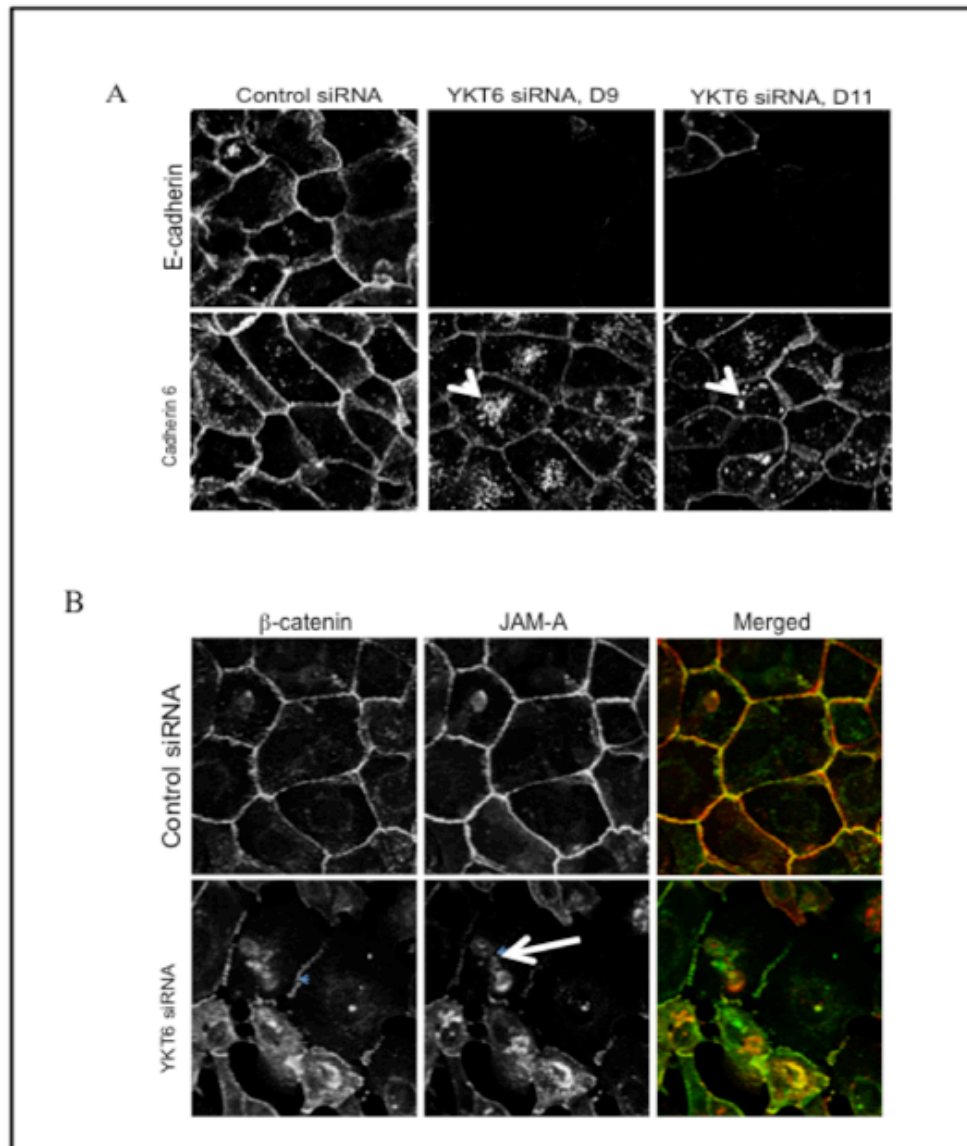


Figure 7: Ykt6 depletion causes Junctional disruption in Du145 cells. 7A. Immunofluorescence staining for Control and Ykt6-knockdown Du145 Cells performed on the 4th day post-transfection. E-cadherin expression is depleted in the Ykt6 knockdown cells. The amount of Cadherin-6 is seen to decrease from the Junctions and in turn accumulate in various intracellular vesicles of the cells in Ykt6 knockdown cells (arrowheads). 7B. Immunofluorescence staining for Control and Ykt6-knockdown Du145 Cells performed on the 4th day post-transfection. Control Du145 Cells show a normal staining pattern for the JAM-A protein while the Ykt6 knockdown cells show a disruption in the JAM-A staining pattern of the cells (arrow).

3.4. Hepatocyte growth factor induced junctional disassembly is accelerated by Ykt6 depletion.

DU145 cells represent a good model to study junctional disassembly triggered by proto-oncogenic and pro-inflammatory stimuli. Specifically, these cells undergo AJ/TJ disassembly and scattering after stimulation with hepatocyte growth factor (HGF). Therefore, we next investigated if loss of YKT6 can also modulate stimuli-induced junctional disassembly. Du145 cells were transiently transfected with either control or YKT6 specific siRNA. On day 3 post-transfection, the cells were treated for 24 h with either vehicle or HGF (25 ng/ml). Cells were fixed and immunolabeled for E-cadherin (red) to visualize AJs and a cytoskeletal protein Myosin IIB (green) to evaluate alterations in cell shape. In control siRNA-treated cells, HGF induced AJ disassembly that was manifested by discontinuous junctional labeling of E-cadherin and accumulation of this junctional protein within large intracellular vacuoles (Figure 8: Top panel). Nevertheless, such HGF treated cells remained well-spread and attached to each other. Remarkably, HGF treatment caused different effects on YKT6-depleted DU145 cells. These cells not only lost E-cadherin labeling but also disassembled the majority of cell-cell contacts with their neighbors and appeared as an array of spindle-shaped separated cells (Figure 7B: Bottom panel). Together these observations indicate that YKT6 depletion not only results in disruption of steady-state-AJs and TJs but also dramatically accelerates stimuli-induced disassembly of epithelial junctions.

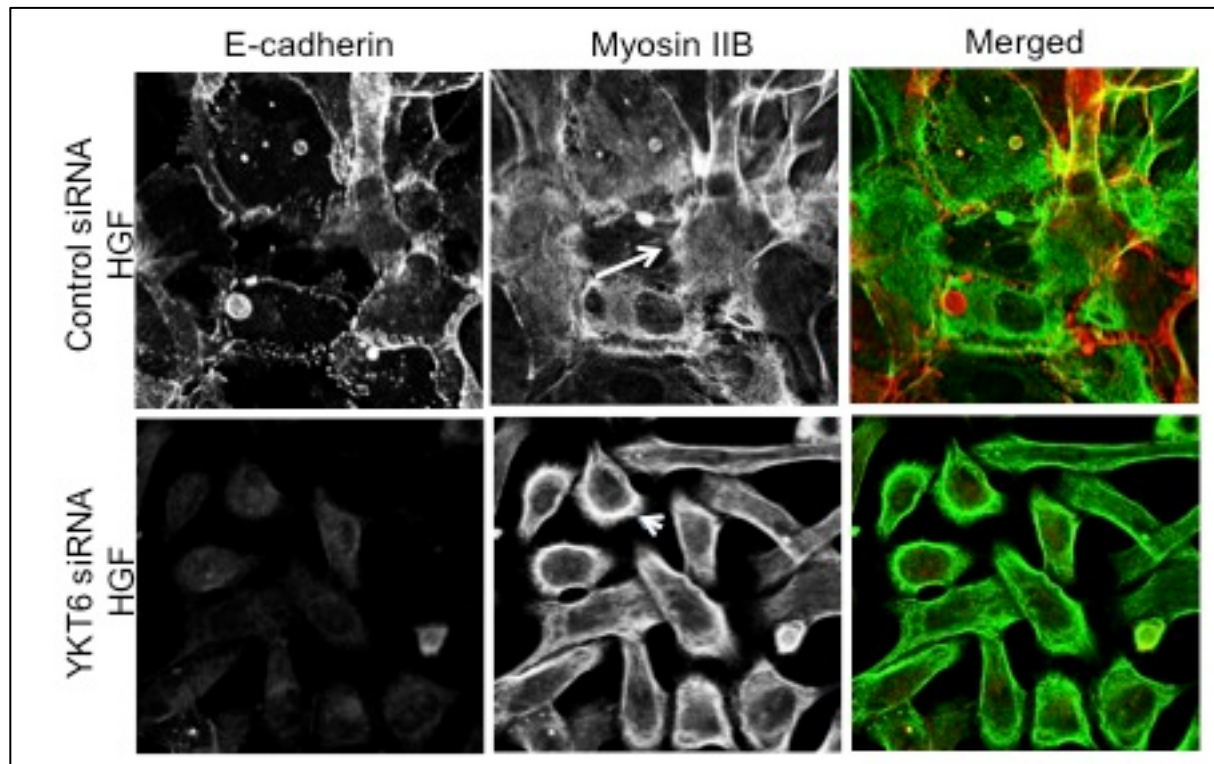


Figure 8: Ykt6 depletion accelerates HGF-induced junctional disassembly.

Du145 Cells were transiently transfected with Control or Ykt6-specific SiRNA. Control and Ykt6 depleted Du145 cells were treated with Hepatocyte growth Factor (HGF) on the day 3 post transfection. The cells were fixed and labeled with junction and cytoskeleton specific antibodies like E-cadherin (red) and Myosin IIB (green). HGF induced junctional disassembly was observed in control cell (arrow) treated with HGF [Top Panel] while a complete cell scattering was characteristic for the Ykt6 knockdown cells (arrowhead) [Bottom Panel].

3.5. Ykt6 depletion alters the expression of certain junctional proteins.

Given our microscopy data revealing decreased intensity of E-cadherin labeling in Ykt6 depleted epithelial cells, we next sought to quantify the effects of Ykt6 depletion on the expression of different AJ and TJ proteins. Du145 cells were transiently transfected with control or Ykt6 specific SiRNA and were harvested for cell lysates on the 4th day post transfection. Immunoblotting analysis revealed significant effects of Ykt6 depletion on expression of different junctional proteins (Figure 9A). Specifically, the level of E-cadherin and Claudin-4 decreased

approximately 2 fold compared to control. The level of JAM-A was increased by around 4 to 7 fold (Figure 9B). The expression of other AJ and TJ proteins appeared to be unaffected.

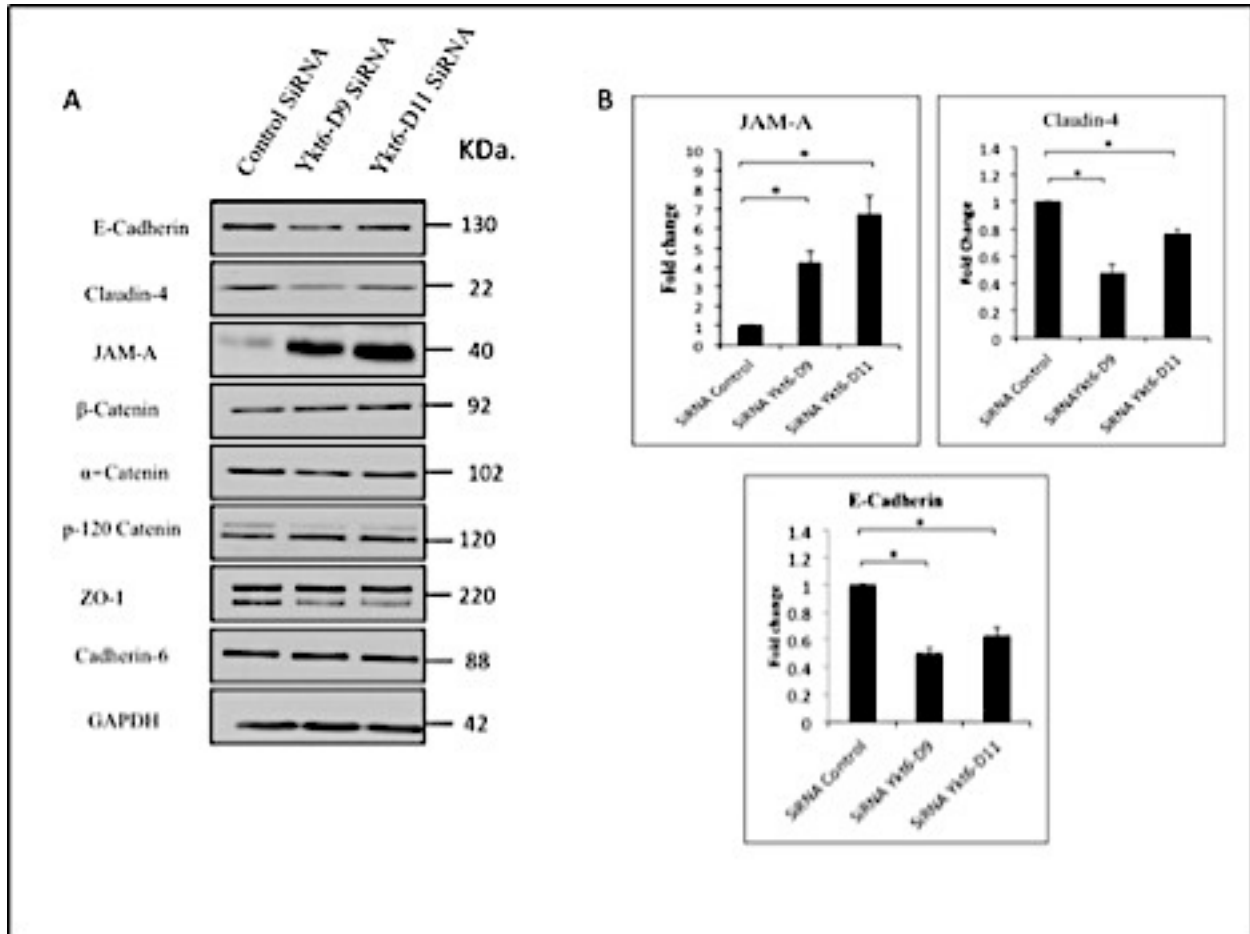


Figure 9: Ykt6 depletion affects expression of different junctional proteins. Du145 Cells were transiently transfected with Control or Ykt6-specific SiRNA. Immunoblotting was performed for Junctional Proteins on cell lysates obtained on the 4th day post transfection. Figure 9A. Shows the Immunoblots for junctional protein expression while Fig.9B shows the quantitative values for the junctional proteins that show a difference in their protein expression. Student's unpaired t-test was used to determine whether the change in protein expression of the three junctional proteins was significantly different. (* $p < 0.01$).

Overall, this part of the study reveals for the first time that YKT6 is essential for the integrity of AJs and TJs in DU145 epithelial cells and it suppresses junctional disassembly and scattering of

HGF-treated cells. A possible mechanism underlying the effects of YKT6 on epithelial junctions involves regulation of expression of certain AJ and TJ proteins. Further studies are planned to dissect molecular events involved in YKT6-dependent regulation of epithelial junctions.

4. RESULTS- PART II

4.1. Depletion of Ykt6 enhances migration of Du145 cells.

Epithelial cells are known to have a functional cross talk between cell-cell junctions and ECM adhesions. Also, junctional disassembly can affect integrity of matrix adhesion and cell migration. Since our data revealed significant disruption of epithelial AJs and TJs in YKT6-depleted cells (Figure 7), we now sought to investigate if loss of this SNARE protein can also affect cell motility and ECM adhesions. In order to investigate the roles of YKT6 in regulating cell motility we used two different assays, a wound healing assay that depends on collective migration of epithelial sheets and a Matrigel invasion assay that involves migration of individual or small clusters of epithelial cells through a 3-D matrix. We observed that 24 hrs post wounding, control siRNA transfected epithelial cells migrated into the wound to cover approximately 30% of its initial area (Figure 10). Interestingly, DU145 cells transfected with two different YKT6 siRNA duplexes migrated significantly faster resulting in up to 50% of wound closure (Figure. 10).

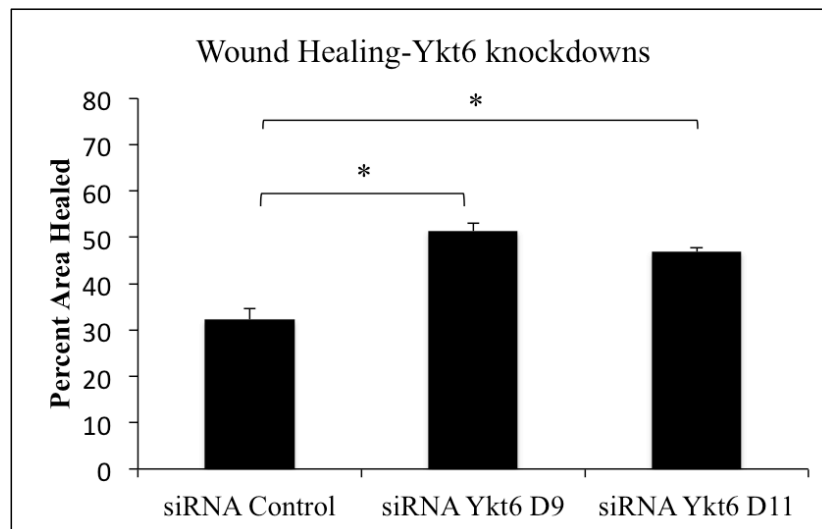
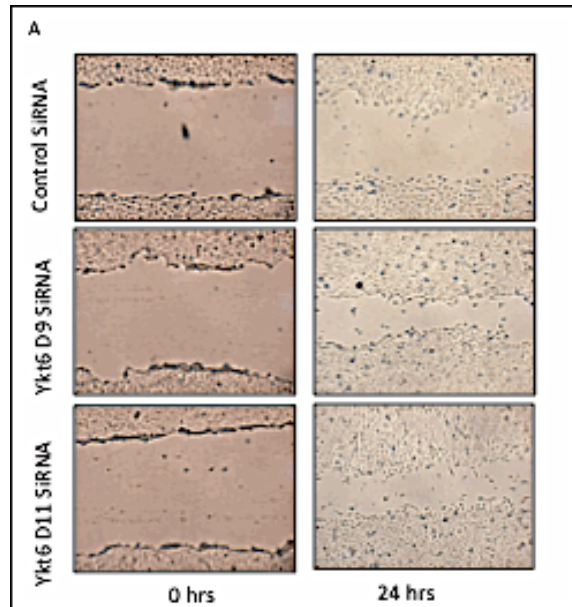


Figure 10: Ykt6 depletion enhances cell migration in Du145 Cells.

Du145 Cells were transiently transfected with Control or Ykt6-specific siRNA. The Wound scratch assay was performed on the 3rd day post transfection. Images were taken at 0 hrs and 24 hrs for comparison of the rate of migration. The results are quantified as Percent open area healed in 24 hrs. Fig. 10A shows representative images of the rate of migration in control cells as compared to the Ykt6 knockdown cells. The bar chart in Fig. 10B shows the quantitation of the observed effect of Ykt6 knockdown on cell migration. Student's unpaired t-test was used to determine whether the change in the rate of migration was significantly different (* $p < 0.01$).

More pronounced effects of YKT6 knockdown was observed by using Matrigel invasion assay. Figure 11 shows that YKT6 depletion by two different siRNA duplexed resulted in dramatic (up to 7 fold) increase in cell invasion (Figure 11). Together these results highlights anti-migratory role of YKT6 in Du145 cells.

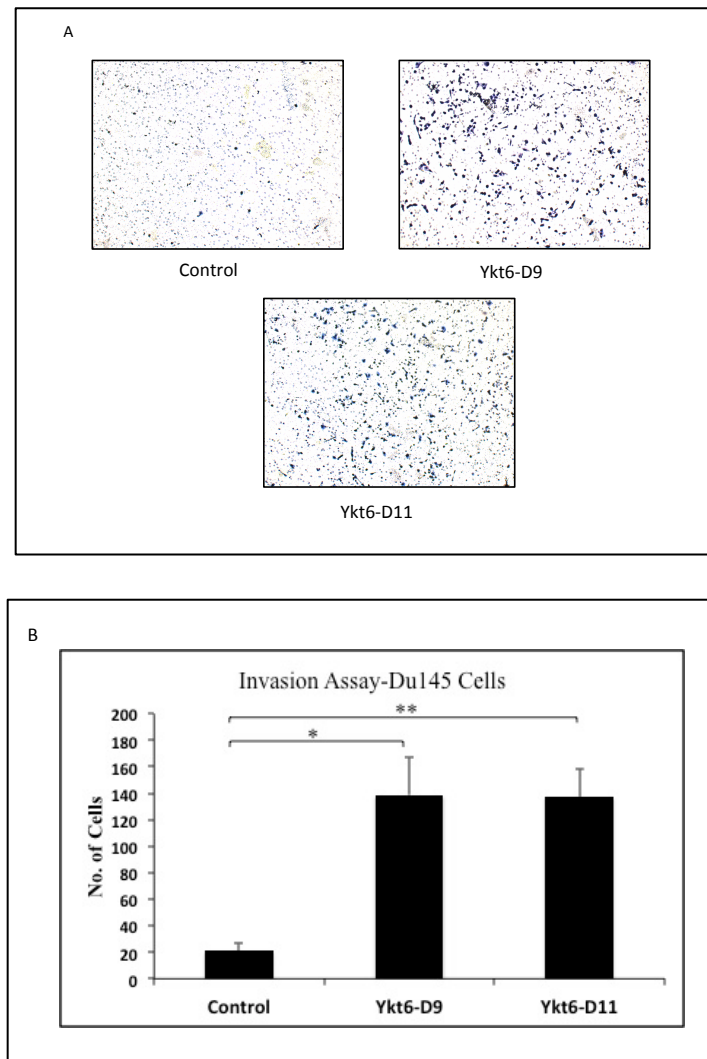


Figure 11: Ykt6 depletion accelerates matrix invasion of Du145 cells.

Du145 Cells were transiently transfected with Control or Ykt6-specific SiRNA. 3×10^7 Cells were loaded onto matrigel wells on the 3rd day post-transfection. The cells were allowed to invade overnight and were subsequently fixed and stained using the DiffQuick staining kit. The figure 11A shows the representative images of the Invasive properties of Ykt6-knockdown cells

as compared to the control. The bar chart in 11B shows the quantitative values of the number of invading cells in the Ykt6 knockdown cells as compared to the control. Student's unpaired t-test was used to determine whether the change in the rate of invasion was significantly different between the control and Ykt6 knockdown groups. (* $p < 0.05$, ** $p < 0.01$).

4.2. Depletion of Ykt6 does not cause an increase in cellular proliferation.

The results above indicated that Ykt6 depletion caused the Du145 epithelial cells to migrate as well as invade faster as compared to control Du145 epithelial cells. Under certain experimental conditions, motility of epithelial cells can depend on cell proliferation. On the other hand, a previous study has implicated YKT6 in regulating proliferation of neuronal cells ^[100]. Hence, it was essential to determine whether loss of YKT6 expression has any effect on epithelial cell proliferation. Accordingly, we carried out the Bivariate BrdU cell proliferation assay on Control and Ykt6 knockdown Du145 cells. The results from the cell proliferation assay are presented in Figure 12 above. The graphs presented in the figure show the distribution of cells in the four cell cycle phases- G1/G2-M/S phase. Proliferating cells are generally observed in the S phase of the cell cycle. As indicated in the figure above, out of the four quadrants, the Lower left section represents the G1 phase while the lower right represents the G2/M phase of the cell cycle. The upper left of the graph represents the S phase of the cell cycle that is the major section of interest for us. We observed that the overall percentage of cells in the S phase in the Ykt6 knockdown cells is pretty close to that in control cells (Figure 12). These results indicate that increase in cell proliferation could not have been responsible for the increase in collective cell migration observed in the wound scratch assay.

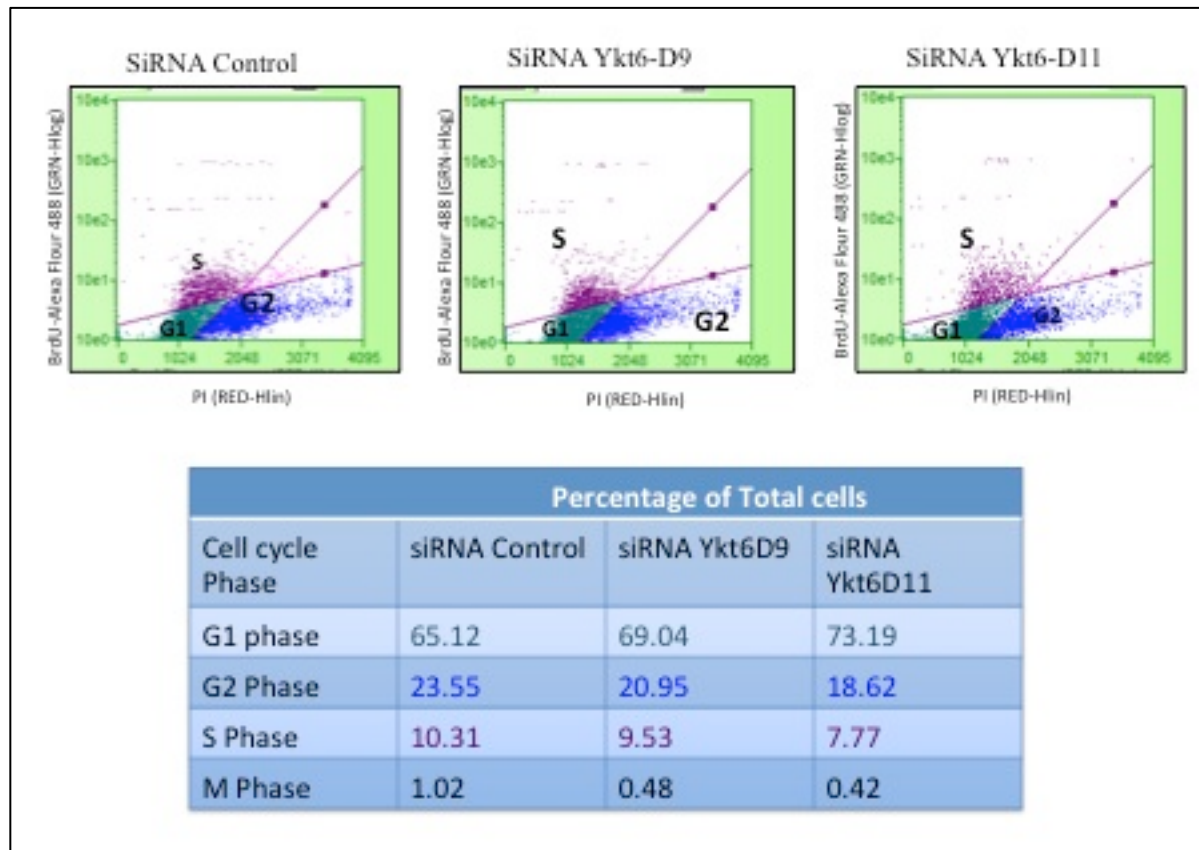


Figure 12: Ykt6 depletion does not cause an increase in cellular proliferation

Du145 Cells were transiently transfected with Control or Ykt6-specific SiRNA. Actively proliferating cells were subsequently pulse labeled with Bromodeoxyuridine (BrdU) on day 4 post transfection for 1hr. The stained cells were flow sorted using the Guava Flow sorter as per the manufacturers protocol. A total of 10000 cells were counted for each of the groups. Note that Ykt6 knockdown did not affect the rate of cell proliferation to any significant effect as compared to the control cells.

4.3. Depletion of the Ykt6 protein increases epithelial ECM adhesion and spreading.

Cell migration is known to be a multistep process involving cell adhesion, cell spreading, retraction and other events. Since ECM adhesion and spreading are two initial and rate-limiting steps of the migration process, we next investigated how these steps are affected by Ykt6

depletion. Figure 13B shows that loss of Ykt6 resulted in up to two-fold increase in Du145 cell attachment to collagen I. Furthermore, Ykt6 depleted cells appear to spread faster over collagen substratum as was manifested by the increased surface area of the attached cells (Figure. 14). Taken together, these data strongly suggests that increased ECM adhesion and spreading mediate enhanced motility of YKT6-depleted epithelial cells.

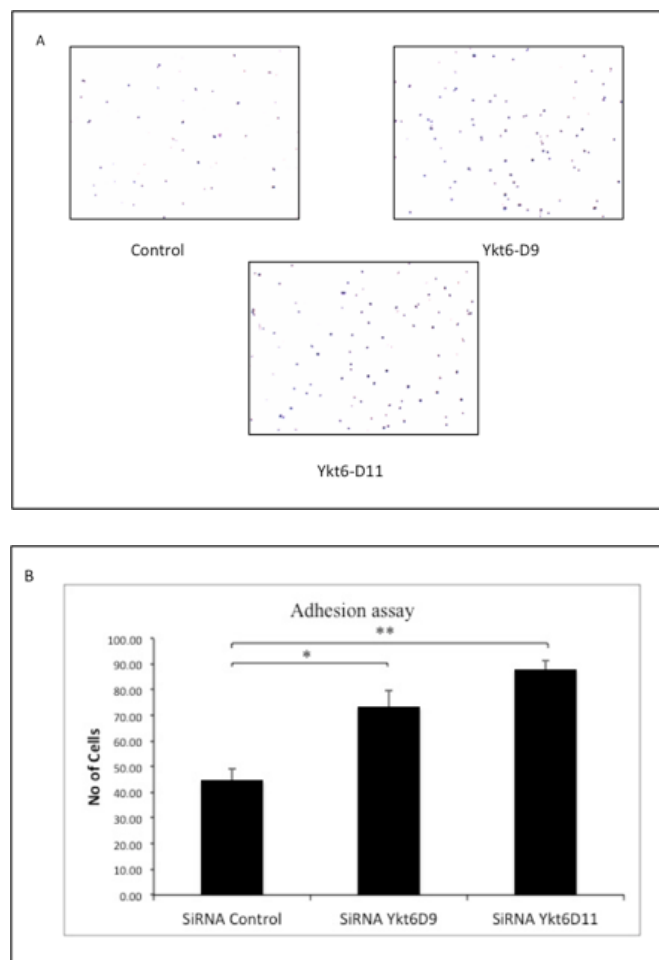


Figure 13: Ykt6 knockdown increases epithelial cell-matrix adhesion.

Du145 Cells were transiently transfected with Control or Ykt6-specific SiRNA. The adhesion assay was performed using Control and Ykt6 knockdown cells on the 4th day post transfection. 10^7 cells were loaded onto collagen coated 24-well plates and were allowed to settle for 30-45

minutes. The cells were then stained using the DiffQuick staining kit. Note that Ykt6 knockdown cells adhered faster to the matrix as compared to the control cells. Fig.13A shows the images for stained control and ykt6 knockdown cells. Fig.13B shows the quantification of the adhesion assay. Student's unpaired t-test was used to determine whether the increase in the adhesive property of Ykt6 knockdown cells was significant as compared to the control cells. (* $p < 0.05$, ** $p < 0.01$).

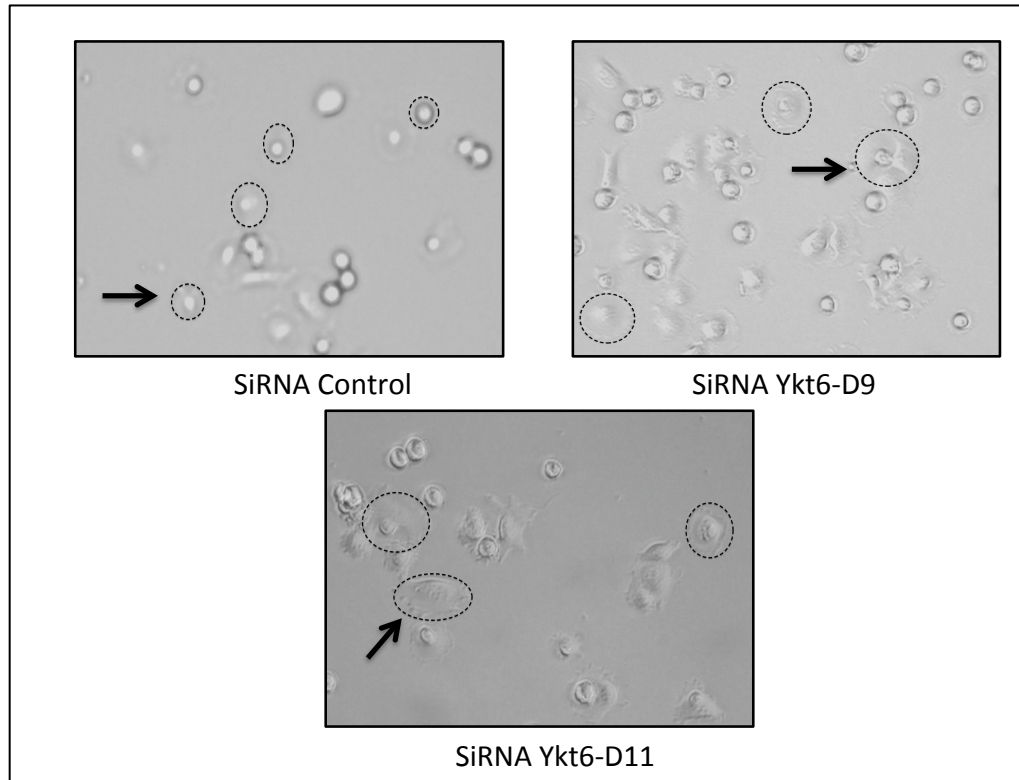


Figure 14: Ykt6 knockdown accelerates cell spreading.

Du145 Cells were transiently transfected with Control or Ykt6-specific SiRNA. The Spreading assay was performed using Control and Ykt6 knockdown cells on the 4th day post transfection. 10^7 cells were loaded onto collagen coated 24-well plates and were allowed to settle for 45 minutes after which they were imaged using high power microscope. Note that the Ykt6 knockdown cells spread faster as compared to the control cells. The dotted circles indicate the spread area of the cells.

Newly attached cells spread over the ECM by forming different types of membrane protrusions.

In order to investigate if YKT6 is directly involved in protrusion formation, we next visualized this SNARE protein in migrating epithelial cells. We overexpressed myc-tagged wild type YKT6

in HEK 293 cells and visualized its localization using an anti-myc antibody. We observed that significant fraction of YKT6 accumulated on protrusive cells edges (Figure 15). We also sought to investigate the effects of certain mutations of YKT6 on intracellular protein localization in spreading cells. Our overexpression data suggests that deletion of the NT domain (deletion of entire N-terminal domain- 1-136 amino acid deletion) or mutations on two lipidation sites of YKT6 molecule (Ykt6 CC194/195 AA mutant) did not affect its accumulation in peripheral protrusions (Figure. 15). In the Ykt6 CC194/195 AA mutant, two of the C-terminal cysteines are mutated into an alanine. Interestingly, overexpression of another YKT6 mutant F391 that bears a mutation in the SNARE domain enhanced formation of peripheral membrane protrusions, whereas a point V8D mutation in the longin domain resulted in mis-localization of YKT6 from membrane protrusions into cytoplasmic vesicles (Figure 15). Together this data suggests that YKT6 is enriched in peripheral membrane protrusions of migrating epithelial cells and can modulate protrusion formation.

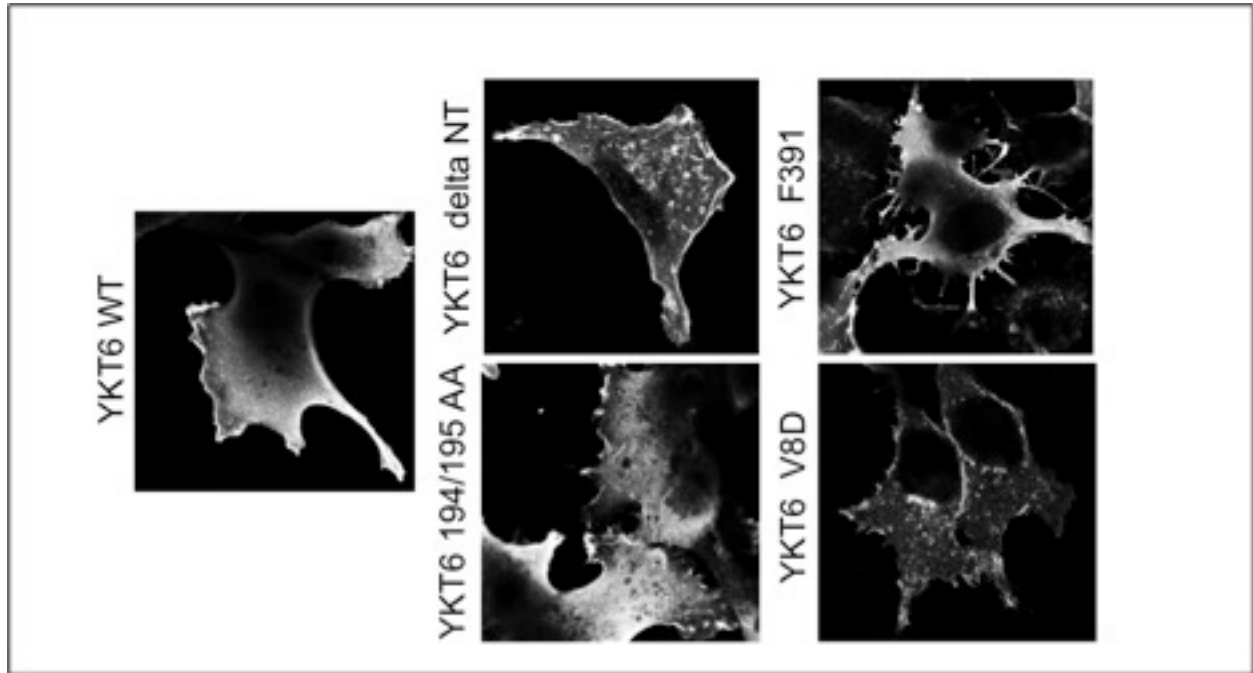


Figure 15: Localization of wild-type and mutant Ykt6 in migrating epithelial cells.

Wild type YKT6 and its several mutants were overexpressed in 293 HEK cells and protein localization in migrating/spreading cells was determined by immunolabeling with Myc-tag antibody. Note prominent localization of wild type YKT6 at the migrating leading edge and different effects of mutations on such localization. For example, F391 mutants promote formation of peripheral protrusions, a V8D mutants disrupts peripheral targeting of YKT6 whereas 194/195 AA mutants and delta NT mutants have similar localization as compared to wild type protein.

4.4. Ykt6 depletion enhances the assembly of focal adhesions at the migrating leading edge.

Assembly of FA regulates ECM adhesion and spreading of epithelial cells. To test the effect of YKT6 depletion on FA assembly, we wounded control and Ykt6 depleted Du145 cell monolayers and visualized FA formation at the wound edge using immunolabeling for a common FA scaffolding protein, vinculin. We observed that both control and YKT6-depleted cells form well-defined elongated vinculin-based FA at the leading edge (Figure 16). On closer observation, it appears that FA in YKT6-depleted cells are large compared to similar structures

in control siRNA-transfected cells, which may reflect greater stability and adhesiveness of these complexes. This observation is in accordance with our data on the adhesive property of Ykt6 depleted cells.

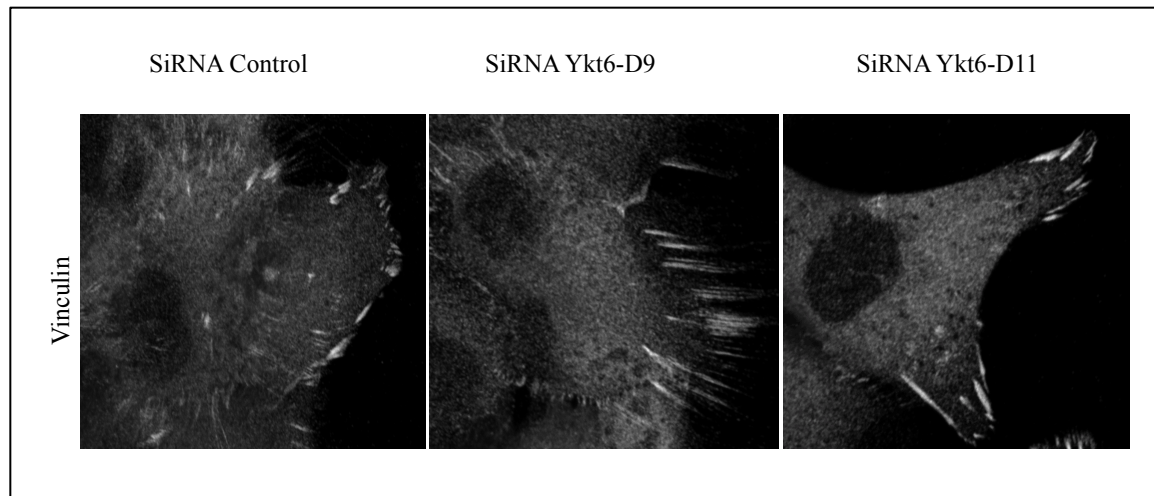


Figure 16: Ykt6 depletion accelerates assembly of focal adhesion at the migrating leading edge.

Du145 Cells grown on coverslips were transiently transfected with Control or Ykt6-specific SiRNA. A wound was made on the coverslips on the 3rd day post transfection. 8hrs after the wound was made; the migrating cells were fixed and stained for vinculin. Note that the vinculin staining pattern shows elongated FA at the leading edge in the Ykt6 knockdown cells as compared to the control cells.

4.5. Depletion of Ykt6 selectively affects the biogenesis of certain focal adhesion proteins.

To further evaluate the effect of Ykt6 depletion on regulating Cell-matrix adhesions, it was important to check the effect of Ykt6 depletion on different important cell-matrix adhesion proteins. Accordingly, Du145 cells were transiently transfected with control or Ykt6 specific SiRNA. On the 4th day post-transfection, cell lysates were obtained and the samples were analyzed for the expression of different focal adhesion proteins. Immunoblot analysis indicated

that depletion of Ykt6 selectively affects the protein levels of different focal adhesion proteins (Figure 17A).

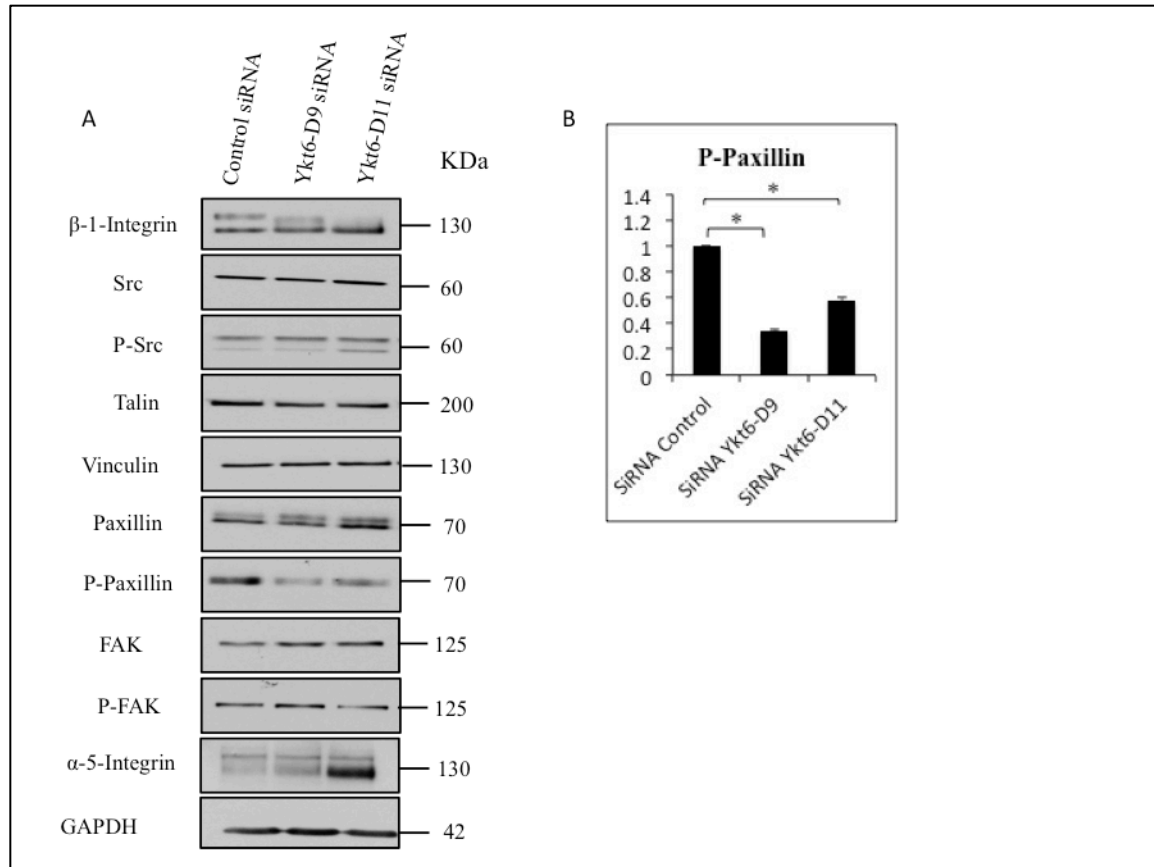


Figure 17: Ykt6 depletion affects biogenesis of several Focal Adhesion proteins.

Du145 Cells were transiently transfected with Control or Ykt6-specific SiRNA. Immunoblotting was performed for Focal adhesion Proteins on cell lysates obtained on the 4th day post transfection. Figure 17A. Shows the Immunoblots for focal adhesion protein expression while Fig.17B. shows the quantitative values for the focal adhesion proteins that show a difference in their protein expression. Note that the maturation of B-1-integrin is blocked due to Ykt6 knockdown while the expression of p-Paxillin is found to be Downregulated. Student's unpaired t-test was used to determine whether the change in protein expression of the focal adhesion proteins was significantly different. (* $p < 0.01$).

Consistent with the observation that depletion of Ykt6 did not severely affect the assembly of focal adhesions in migrating cells, a majority of the focal adhesion proteins were found to be unaffected by Ykt6 knockdown. However, a very important focal adhesion protein from the integrin family i.e. the β -1-integrin protein was significantly affected due to Ykt6 knockdown. The β -1-integrin protein when analyzed through western blotting generally presents two bands- a lower band which is the immature, un-glycosylated band (pre- β -1-integrin) and the upper band which is the mature glycosylated version of the protein. Indeed, the Immunoblot analysis of control Du145 cells detected a major β -1-integrin doublet. By contrast, loss of Ykt6 resulting in the loss of the upper glycosylated β -1-integrin band indicating that loss of Ykt6 impairs the maturation of the important focal adhesion protein integrin. Accordingly, we ran a few other important integrin proteins to check whether any of them compensate for the loss of mature β -1-integrin or show the same effect as β -1-integrin. Our Immunoblotting results indicated that at least one other integrin protein i.e. α -5-Integrin is upregulated in Ykt6 depleted cells (Figure 17A). However, this effect was not found to be consistent between the two Ykt6 duplexes.

Another significant observation from this experiment was the evident dephosphorylation of the Paxillin protein, which is an important signaling FA protein. The Paxillin protein serves as a docking protein for the recruitment of Focal adhesions and specific combinations of signaling molecules in a complex to coordinate downstream signaling that ultimately regulates cell spreading and cell motility ^[94]. Interestingly, both of these mechanisms were found to be affected in Ykt6 depleted cells, suggesting that Paxillin expression may in some way be responsible for the effect of Ykt6 depletion observed on Cell migration in Du145 cells.

4.6. Ykt6 depletion induces Golgi fragmentation in different epithelial cells.

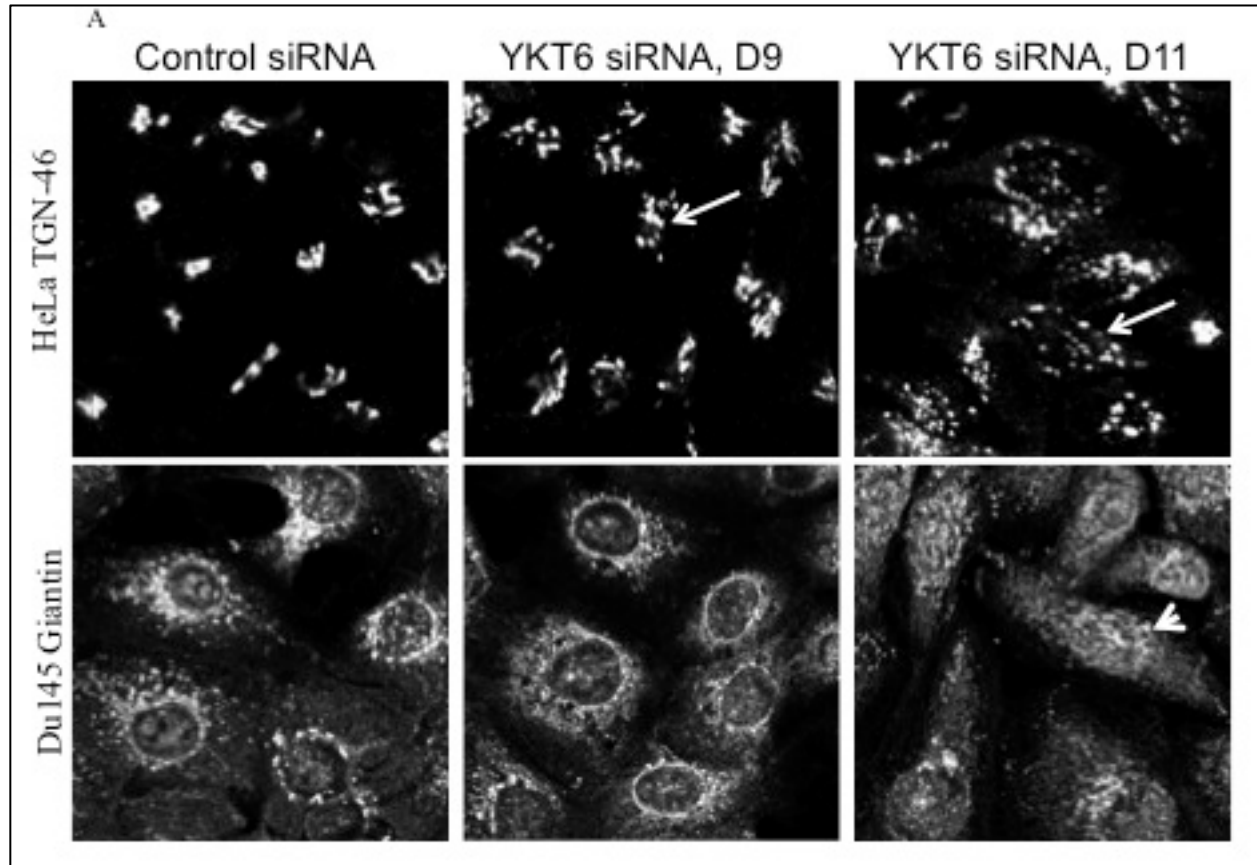


Figure 18: Loss of Ykt6 induces Golgi fragmentation in different epithelial cells.

Du145 Cells were transiently transfected with Control or Ykt6-specific SiRNA. HeLa Cells and Du145 cells were fixed and labeled with Golgi specific antibodies like TGN-46 and Giantin. Note that Ykt6 knockdown cells in both the epithelial cell lines show a disruption in their Golgi staining pattern.

Prior studies have suggested that Golgi-dependent events such as glycosylation and trafficking of β -1-integrin seemingly play a very important role in the regulation of FA ^[76]. Moreover, through our previous experiment, we determined that Ykt6 depletion might block the maturation of the β -1-integrin protein. It is a known fact that this protein undergoes Glycosylation (maturation) in the Golgi before being finally transported to the plasma membrane. Also, previous studies by our lab

and their collaborators have also indicated that disruption of the Golgi apparatus leads to disruption of the maturation of the focal adhesion proteins. Hence, we proposed to observe the effect of Ykt6 knockdown on the Golgi apparatus, in order to assess whether the same effects are observed even in this case. Accordingly, Du145 cells and HeLa Control and Ykt6 depleted cells were analyzed for Golgi specific antibodies like TGN-46 which is a trans-Golgi marker (HeLa cells) [Fig.18-Top panel] and Giantin that is a cis-Golgi marker (Du145 cells) [Fig.18-Bottom panel]. Our observations indicated that depletion of Ykt6 caused fragmentation of the Golgi apparatus in both of these epithelial cell lines, suggesting that loss of Ykt6 may inhibit the trafficking of adhesions proteins from the Golgi to the plasma membrane.

4.7. Loss of E-cadherin, β -1-integrin and Paxillin does not increase collective migration of Du145 cell monolayers.

Since our previous experiments indicated that loss of Ykt6 significantly depleted the total protein levels of E-cadherin and p-Paxillin while seemingly blocking the maturation of the β -1-integrin protein (Figure 9 & Figure 17), we proposed to assess whether anyone of these proteins was the main player in the mechanism involved in accelerating the migration potential of the Du145 cells. Comparison of the knockdown groups for E-cadherin, β -1-integrin and Paxillin with control groups indicated that the collective migration of Du145 epithelial cells was not significantly affected by the depletion of either E-cadherin or Paxillin after 24 hrs of Wounding (Figure 15B & 15D). Additionally, the knockdown down of β -1-integrin showed a slight decrease (15%) in the collective rate of migration in the Ykt6 depleted cells after 24 hrs of wounding (Figure 15 D). Hence, none of the above experiments showed similar effects as knockdown of Ykt6 protein in epithelial cells thereby making it unlikely that any of the above

three proteins are directly involved in increasing the rate of epithelial cell migration in Ykt6 depleted cells.

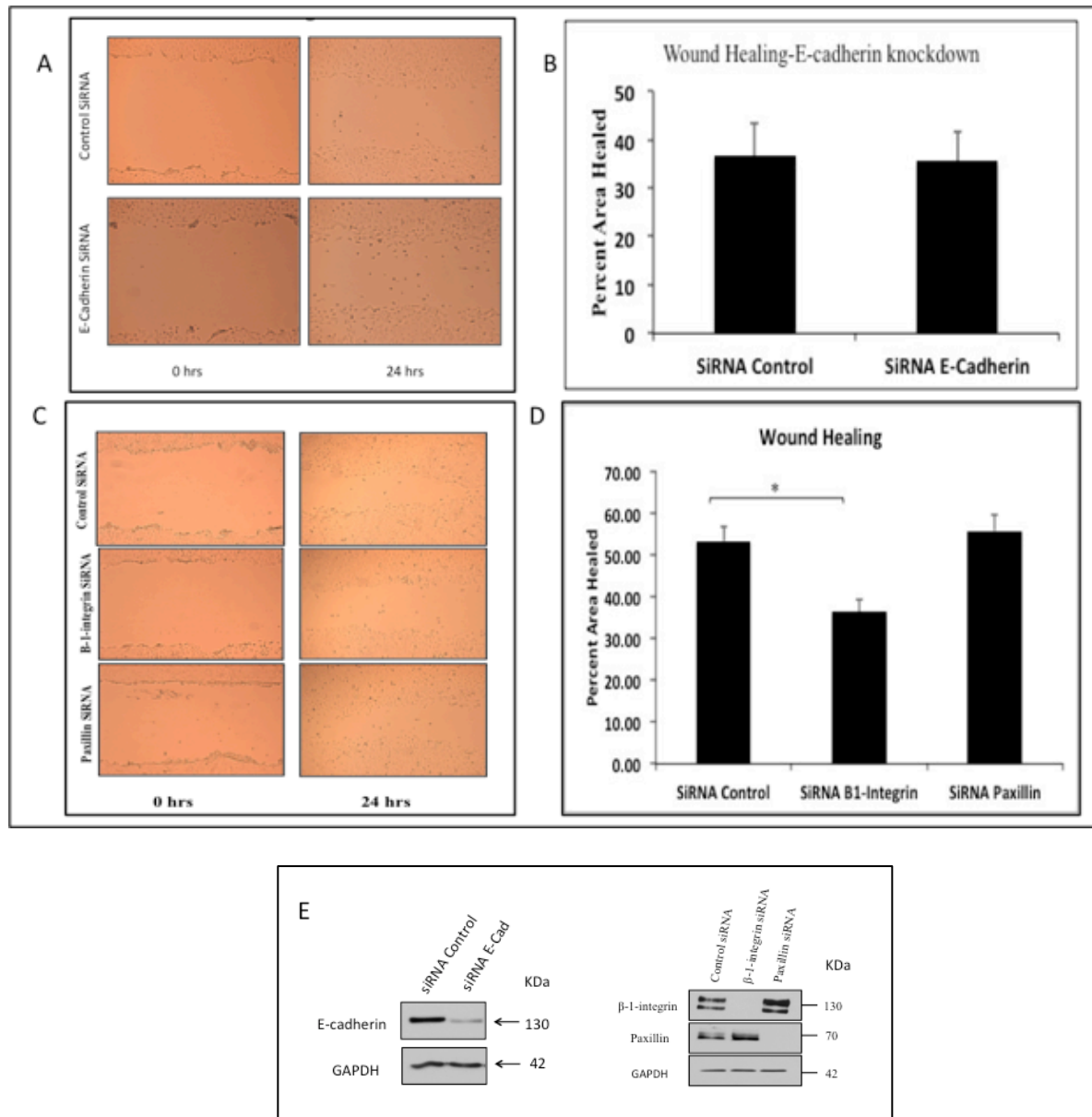


Figure 19: Knockdown of E-cadherin/β-1-integrin/Paxillin does not increase the rate of collective cell migration in Du145 cell monolayers.

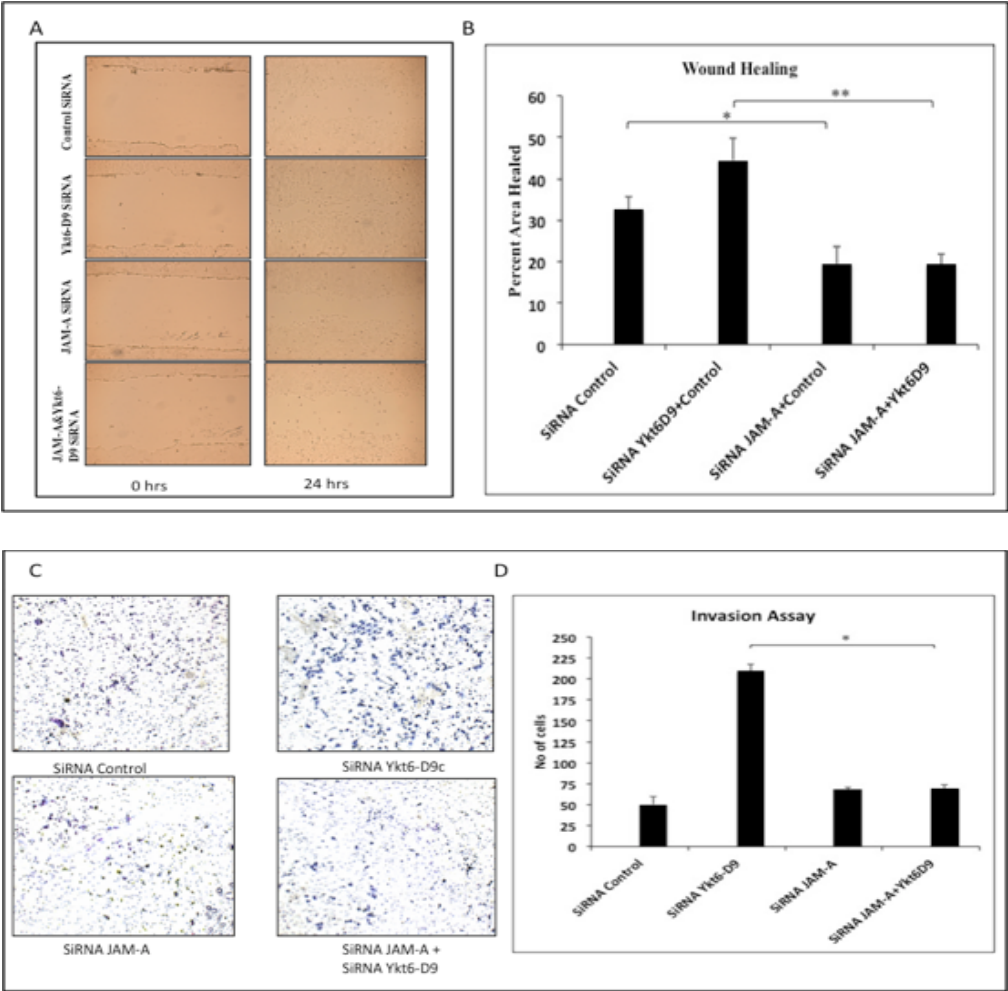
Du145 Cells were transiently transfected with Control or E-cadherin/Paxillin/β-1-integrin specific SiRNA. The Wound scratch assay was performed on the 3rd day post transfection.

Images were taken at 0 hrs and 24 hrs for comparison of the rate of migration. The results are quantified as Percent open area healed in 24 hrs. Fig. 19A & 19C shows representative images of the rate of migration in control cells as compared to the knockdown cells. The bar graph in Fig. 19B & 19D shows the quantitation of the observed effect of the knockdown on cell migration. Student's unpaired t-test was used to determine whether the change in the rate of migration was significantly different. (* $p < 0.01$). Fig. 19E the successful knockdowns of the all the groups under study

4.8. Depletion of JAM-A reverses the effect of Ykt6 knockdown on collective migration and invasion of Du145 Cells.

Ykt6 depletion led to a significant up-regulation in the level of the JAM-A protein as compared to control cells (Figure 9). Moreover, our studies also showed a significant increase in epithelial cell migration upon Ykt6 depletion (Figure 10). The JAM-A protein has previously been found to regulate cell migration in certain Breast cancer cells through the Rap1 GTPase pathway ^[94]. Therefore, we hypothesized that JAM-A could be the contributing mechanism to the functional effects such as collective cell migration and cell invasion observed in the Ykt6 depleted cells. To test this hypothesis, we carried out the Wound Scratch assay and the Invasion assay on Ykt6 and JAM-A co-knockdown cells in order to observe whether the effect of Ykt6 depletion is rescued due to the knockdown of the JAM-A protein. Firstly, JAM-A single knockdown was found to reduce 24hrs wound closure by almost 20% as compared to the control although no significant difference was found between cell invasion in JAM-A depleted and Control cells. Next, we observed that co-knockdowns of Ykt6 and JAM-A reduced the rate of cell migration (Figure 20.A & B) almost 25% compared to that of Ykt6 single knockdowns. In fact, the levels of collective migration in the co-knockdowns were found to be similar to the cell migration in JAM-A single knockdown cells. Furthermore, co-knockdowns of Ykt6 and JAM-A reduced the rate of cell invasion almost 25% compared to that of Ykt6 single knockdowns (Figure 20.C & D)

strengthening our theory that JAM-A could be the possible mechanism through which faster rate of invasion and migration is seen in Ykt6 depleted cells.



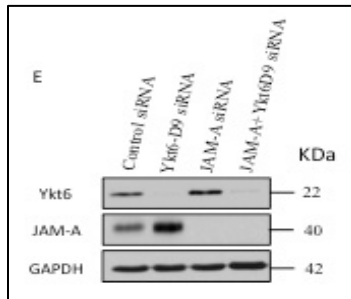


Figure 20. Depletion of JAM-A reverses the effect of Ykt6 knockdown on collective migration and invasion of Du145 Cells.

Du145 cells were transiently transfected with control(100nm)/ Ykt6-D9/JAM-A or Ykt6-D9 + JAM-A specific SiRNA. The Wound scratch assay was performed on the 3rd day post transfection. Images were taken at 0 hrs and 24 hrs for comparison of the rate of migration. For invasion, 3×10^7 Cells were loaded onto matrigel wells on the 3rd day post-transfection. The cells were allowed to invade overnight and were subsequently fixed and stained using the DiffQuick staining kit. 20A & 20C show representative images from Wound scratch and Invasion experiments while 20B & 20D are the quantified bar-graphs of the Wound Scratch and Invasion respectively. Student's unpaired t-test was used to determine whether the change in the rate of migration or invasion was significantly different. (* $p < 0.05$, ** $p < 0.01$). Fig.20E shows the successful knockdowns of the all the groups under study.

5. DISCUSSIONS

Vesicular trafficking (consisting of endocytosis and exocytosis) has been shown to be important for the regulation of both cell-cell as well as cell-matrix adhesions thereby contributing to the maintenance of epithelial integrity. For instance, Endocytosis is known to be required for the recycling of the Focal adhesion proteins as well as epithelial junction proteins ^[37,40,44,45,46,61,62]. Like endocytosis, exocytosis has been also been implicated in the formation and maintenance of epithelial barriers as indicated by the steady state exocytosis of occludin and E-cadherin observed in various epithelial monolayers as well as disruption of the reformation of the Tight and Adherens junctions due to disruption of exocytosis ^[49-56]. Our studies have identified the membrane fusion protein Ykt6 as a novel regulator of epithelial junction, ECM adhesions as well as cell migration.

Depletion of Ykt6 in Du145 epithelial cells led to junctional disassembly indicating that Ykt6 is a positive regulator of epithelial junctions (AJs and TJs). On the other hand, depletion of Ykt6 promoted the attachment of epithelial cells to its ECM and also accelerated cell migration indicating that Ykt6 is a negative regulator of ECM adhesions and cell migration.

5.1. Ykt6 and its effect on assembly of epithelial junctions.

One of the major findings of this study was the disruption of cadherin-based junctions in Du145 epithelial cells on the depletion of the Ykt6 protein (Figure 7). Our studies on the localization of the Ykt6 protein in HEK293T cells indicated that Ykt6 is localized close to the nucleus and also

in the cell-cell junctions (Figure 5). Also, our analysis of the expression levels of specific junctional proteins indicated that E-cadherin as well as Claudin-4 levels significantly decreased in Ykt6 knockdown cells as compared to the control cells (Figure 9). This same observation was reiterated in our IF studies, wherein the labeling intensity of E-cadherin at AJs was found to be drastically decreased (Figure 7). Interestingly, our IF studies indicated that junctional localization of Cadherin-6, another epithelium specific classical cadherin, was decreased and this protein accumulated in intracellular vesicles (Figure 7). We hypothesize that one of two mechanisms could be responsible for the observed effects of Ykt6 knockdown on epithelial junctions. Firstly, targeting of cadherin or Claudin-4 from the Trans golgi network (TGN) to the plasma membrane could have been inhibited leading to some of the protein being mis-targeted for degradation and some being retained in the endosomes. It is possible that E-cadherin and Claudin-4 were mistakenly targeted for degradation while Cadherin-6 was retained in the endosomal compartments or mis-targeted. Further studies need to be performed to confirm whether this mechanism is the one responsible for the depletion of specific junctional proteins and accumulation of Cadherin-6 in Ykt6 depleted cells.

It is known that E-cadherin is transported from the site of its synthesis (ER) to the Plasma membrane by a specific vesicle trafficking pathway ^[95]. It first exits the ER following which it gets sorted into the TGN. Next, it passes the recycling endosomal compartment and finally comes the docking and fusion of the protein with the plasma membrane. The transport of the Cadherins from the ER to the Golgi and its fusion with the Golgi membrane is facilitated by the SNARE proteins as was already explained in Chapter 1. Our studies have shown that Ykt6

depletion led to the fragmentation of the Golgi in different epithelial cell lines (Figure 18). Golgi fragmentation can lead to the disruption of the ER-Golgi transport pathway further leading to a down-regulation in the E-cadherin and Claudin-4 protein levels and intracellular accumulation of Cadherin-6 in the Ykt6 depleted epithelial cells.

Another significant finding from this study was the up-regulation of JAM-A in response to Ykt6 depletion (Figure 9). However, the mechanism of Ykt6 induced upregulation of the JAM-A protein is still unknown. Interestingly, recent studies have indicated that apart from being integral in exocytosis related fusion events, Ykt6 could also be involved in endocytosis from the plasma membrane and retrograde trafficking of molecules from the endosomal compartments to the TGN ^[104]. Since a significant pool of YKT6 localizes at the plasma membrane (Figure 5) it is reasonable to suggest that YKT6 controls internalization and intracellular fate of some plasma membrane proteins including JAM-A. Assuming that internalized JAM-A can be targeted for degradation, inhibition of JAM-A endocytosis in YKT6-depleted cells would likely result in the increased expression of this protein.

5.2. Ykt6 localization and functions.

The precise localization and function of the SNARE protein Ykt6 has been under debate for a considerable period of time. Various groups of researchers have stated that it localizes primarily in the Golgi whereas other groups have found it to be localized variably in the Golgi, ER and Endosomal compartments ^[80,84,92]. In one particular study, the ‘in vitro ER-Golgi transport assay’ performed by supplementing NRK cells (Normal rat kidney epithelial cells) with increasing concentrations of antibodies against Ykt6 led to the complete disruption of the ER-Golgi

transport pathway ^[92]. In a similar study, wild-type endogenous Ykt6 was stained along with Golgi specific mannosidase II. This study indicated that Ykt6 only partially co-localized with this protein indicating that Ykt6 localizes in a separate Golgi compartment as that of mannosidase II ^[92]. Further studies indicated that Ykt6 co-localized with the KDEL receptor that is usually enriched in the cis-Golgi region. These studies thus indicated that Ykt6 is probably involved with ER-to Golgi transport and is also localized predominantly in the cis-Golgi. On the other hand, a second study showed that GFP labeled full length Ykt6 localizes only slightly in the Golgi and to a great extent in the nucleus and cytoplasm ^[84].

Our studies indicated that the localization of Ykt6 is not confined to the Golgi but is actually located close to the nucleus and surprisingly enriched at the plasma membrane of epithelial cells (Figure 5). We confirmed this result by specifically staining for Ykt6 as well as for a Golgi-specific protein Sec22b, both of which showed dissimilar cellular localizations. Moreover, Ykt6 was also enriched in the leading edge of moving epithelial cells (Figure 15). Our studies as well as previous studies with Ykt6 localization suggest that Ykt6 still significantly localizes in the ER and Golgi apart from localizing in the cell-cell junctions. Our studies on the localization of Ykt6 as well as its effect on epithelial junctions suggests that it may not only be an ER-Golgi SNARE protein but may also be involved in the maintenance of epithelial integrity.

While the localization of Ykt6 is still under considerable discussion, one factor that is appearing to be clearer is its importance in the maintenance of the Golgi in different epithelial cells. For instance, in a previous study, treatment with antibodies against Ykt6 caused Golgi fragmentation in L2 Rat epithelial cells ^[92]. The result was further validated by treatment of the same cells with

control antibodies that had no effect on Golgi morphology. In our study, Ykt6 depletion in HeLa and Du145 cells led to Golgi fragmentation (Figure 18). This further emphasizes the importance of the Ykt6 protein in regulation of the Golgi in epithelial cells.

5.3. Ykt6 and its effect on cell motility and ECM adhesions.

Depletion of the Ykt6 protein in epithelial cells was found to result in increased wound closure and cell invasion in epithelial cells (Fig.10 & Fig.11). These functional effects were accompanied by an increase in ECM adhesions and an increase in epithelial cell spreading in Ykt6 knockdown cells. Cell-matrix adhesion and cell spreading are the two initial events that regulate velocity of cell migration and it is reasonable to suggest that increased ECM adhesion and spreading of YKT6 depleted cells play causal role in accelerated cell migration. Our observation of the effect of Ykt6 depletion on cell motility is rather unusual due to the fact that it was exactly opposite to what was observed in previous studies with different SNARE proteins. For instance, knockdown other SNARE proteins like Syntaxin 3 and Syntaxin 4 were observed to inhibit ECM adhesions as well suppress chemotactic induced cell migration ^[101]. This was accompanied by a diminished cell surface expression of integrin proteins. On the other hand, in our study, we observed that Ykt6 depletion led to an increase in cell migration and invasion. These observations further underline the uniqueness of the Ykt6 protein.

However, on the molecular level increased adhesiveness of YKY6-depleted cells is difficult to explain. It is unlikely to be mediated by increased expression or activation of focal adhesion proteins. In fact, our results indicate the opposite effects of YKT6 knockdown that inhibited Golgi-dependent maturation (glycosylation) of β -1-integrin and de-phosphorylation of paxillin (Fig.17). Thus, we proposed that it could be possible that various other integrin subunits are

upregulated in Ykt6 knockdown cells, as a compensatory mechanism to overcome the loss of β -1-integrin protein and to explain the results obtained in the wound healing assay. Accordingly, we assessed our total cell lysates for other integrin molecules and found that at least one other integrin protein i.e. α -5-Integrin is upregulated in Ykt6 depleted cells (Figure 17A). However, this effect was not found to be consistent between the two Ykt6 duplexes and is thus unlikely to explain pro-migratory effects of YKT6 knockdown.

Likewise our suggestion that dysfunction of β -1-integrin in YKT6-depleted cells can be responsible for stimulation of cell motility by upregulating some yet to be defined mechanisms was not supported by experiments with β -1-integrin depletion. Indeed this experiment demonstrated attenuated cell motility following β -1-integrin knockdown in YKT6 cells thereby indicating essential and poorly compensable role on this integrin subunit in motility of prostate epithelial cells. It is likely therefore that YKT6 depletion activates some powerful pro-migratory events that could even override some defects in integrin processing and functions.

This notion is supported by our observation of slightly enhanced FA complexes in YKT6-depleted cells (Fig.16). This happened in spite of loss of phosphorylation of an important FA scaffolding protein, Paxillin. Furthermore, our migration studies with Paxillin knockdown suggests that it is not involved in regulating cellular migration in the Ykt6 depleted cells. However, though Paxillin is the most commonly known regulator of ECM adhesion, it is not the only protein mediating FA assembly. A relatively new family of FA scaffold protein called Parvins, specifically β -parvin (Affixin) may be maintaining Focal adhesions in a Paxillin independent manner ^[102]. However, contrary to this evidence, more recent evidence has

suggested that β -parvin along with other members of the Parvin family of FA scaffolding proteins do bind to Paxillin directly ^[103]. Hence, these observations suggests that though Parvins might not work in a Paxillin independent manner, it is possible that there are other unidentified scaffold proteins that may regulate the assembly of focal adhesion in a Paxillin independent manner.

5.4. Possible involvement of JAM-A protein in the control of cell motility in Ykt6 knockdown cells.

Since our data suggested that neither the inhibition of β -1-integrin processing nor the dephosphorylation of Paxillin was involved in regulating ECM adhesion and motility in Ykt6 depleted cells, we sought to explore alternative mechanisms that can explain these events. Earlier studies have indicated that epithelial cell junctions and cell-matrix adhesions are engaged in a functional cross-talk that can regulate cell motility especially in cancer cell lines. For instance, Integrin-mediated changes in the actomyosin skeleton can lead to the loss of E-cadherin further leading to the loss of cell-cell adhesions ^[105,106]. Hence, we next wanted to assess whether disassembly of epithelial junctions could be responsible for the increased ECM adhesion and motility of Ykt6 depleted epithelial cells. Since depletion of junctional E-cadherin was the most prominent effect of YKT6 knockdown on epithelial junctions, we investigated if loss of E-cadherin can be responsible for pro-migratory effects of YKT6 depletion. However, our experiments with E-cadherin knockdown did not support an idea that disruption of intercellular junctions underlies increased motility of YKT6-depleted epithelial cells.

We then turned our attention to another significantly affected junctional protein i.e. JAM-A. Since JAM-A is known positive regulator of cell motility ^[94] and it is upregulated in Ykt6

depleted cells, we hypothesized that JAM-A might be playing an important role in the control of cellular motility in Ykt6 depleted cells. Accordingly, we transiently transfected Du145 cells with JAM-A specific siRNA and repeated the wound scratch assay on these cells. We observed that JAM-A knockdown decreased the rate of collective cell migration in Du145 cells as compared to the control. To assess whether JAM-A knockdown could reverse the effect of Ykt6 depletion in Du145 cells, we performed co-knockdowns of JAM-A and Ykt6 in this cell line and repeated the wound scratch assay. Indeed, we observed that JAM-A depletion could actually reverse the effect of Ykt6 depletion in Du145 epithelial cells. Similar results were obtained when we performed the invasion assay in JAM-A and Ykt6 co-knockdown Du145 cells. These results suggested JAM-A might be involved in regulating cell motility in Ykt6 knockdown cells.

Previous studies in mammary epithelial cells have indicated that JAM-A regulates cell motility and migration through activation of Rap1-GTPase. These studies have shown evidence that highly invasive breast cancer cells show higher expression levels of the JAM-A protein. Accordingly, knockdown of JAM-A expression in these cell lines led to a decrease in the rate of cellular migration ^[94]. Rap1 GTPase is a known activator of β -1-integrin, but it can also promote cell motility via integrin-independent mechanisms including effects on the actin cytoskeleton ^[96]. For instance, it is known that Rap1GAP regulates the activity of Rap1 protein. Studies have indicated that depletion of Rap1GAP increases cellular motility as well as suppresses ROCK-mediated actomyosin contractility ^[107]. Our data indicate that the effects of JAM-A induction on motility of YKT6-depleted cells unlikely involve β -1-integrin and future studies are required to delineate key signaling events that drive JAM-A dependent motility caused by loss of YKT6.

5.5. Pathophysiological significance of Ykt6 in regulation of cell-cell and cell-matrix adhesions.

It has been established through various studies that Ykt6 is a unique SNARE protein that is potentially involved in multiple cellular mechanisms. One study indicated that Ykt6 is significantly upregulated in certain breast cancers ^[81]. However, close observation of Ykt6 expression in a cancer microarray database “Oncomine” suggested that Ykt6 is actually downregulated in breast cancer, prostate cancer and leukemia. Generally, Ykt6 expression appears to be very variable and can be either up or down-regulated (at least at the mRNA level) in different cancers. This seems to be in accordance with our study that suggests that Ykt6 downregulation increases invasive potential of epithelial cells. Hence, Ykt6 depletion may contribute to the metastatic potential of tumors that have a significant downregulation of Ykt6 expression. Nevertheless, it is evident that further studies need to be performed to assign definitive functions to the Ykt6 protein.

5.6. Future Directions

A major observation of our study is the possible role of JAM-A in regulation of cellular invasion and migration in epithelial cells. This finding needs to be validated and confirmed through in depth experiments. For instance, we have proposed that JAM-A may be controlling cell motility through a Rap1 GTPase dependent mechanism. Inhibitors against Rap1 can be used to check whether the effect of Ykt6 depletion is reversed by inhibition of the Rap GTPase molecule. Further, Claudin-4, another important junctional protein, was also found to be significantly down regulated in Ykt6 depleted cells. Hence, experiments need to be performed to assess whether this

protein is involved in the pathway responsible for the functional effects observed in Ykt6 depleted cells. Further, immunoprecipitation studies can be performed to assess whether any of the aforementioned proteins or E-cadherin are part of the complex involving the Ykt6 protein in different epithelial cell lines.

CONCLUSIONS

In summary, our observations suggest that Ykt6 is an important regulator of cell-cell and cell-matrix adhesions. Our study further validates the findings from previous studies that indicate that Ykt6 is an essential regulator of the Golgi. Finally, our studies have shown that depletion of Ykt6 leads to an increase in rate of cellular migration, cellular invasion, cell adhesion as well as cell spreading. The rate of cell migration and cell invasion could have been increased in Ykt6 depleted cells due to a JAM-A-dependent mechanism. However, further studies need to be carried out to confirm this hypothesis.

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